An investigation of the development of a biochemical and clinical marker for gastric disease

Ms Rufaro Chivaura presented for the degree of Master of Science in Medicine (Surgery)

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To Mum, Dad,

Farai, Rudo and to Fadzai,

with much love.

PLAGERISM DECLARATION

This work was completed under the supervision of Professor A. S. Mall, from 2012 to 2014 in the Division of Surgical Research, University of Cape Town.

I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is one's own. The studies represent the original work by the author and have not been submitted in any other form to another University. Where use has been made of the work of others, it has been duly acknowledged in the text.

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Abstract

An investigation of the development of a biochemical and clinical marker for gastric disease

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Introduction: Gastric cancer is prevalent in the Western Cape region of South Africa and there is no suitable biochemical marker for pre-malignant disease. Previous work from our laboratory showed that a 55-65kDa glycoprotein associated with albumin, reproducibly fractionated with mucins from crude gastric mucus scrapings and gastric juice subjected to ultra-centrifugation in a caesium chloride density gradient. This protein was identified as α-1-acid glycoprotein (AGP) by its protein spots on 2D-SDS-PAGE staining with Coomassie Blue. We sought to confirm the identification of the glycoprotein PAS spot and determine if AGP could be used as a clinical marker for pre-malignant gastric disease. We also determined the levels of MUC1, MUC4, MUC5AC and MUC6 in blood plasma and in gastric tissue (including MUC1c and MUC2), to ascertain if there was any correlation with the levels of AGP.

Methods: Human gastric mucus scrapings were collected after total or partial gastrectomy from Groote Schuur Hospital. Briefly the mucus scrapes were collected into 5.0 ml of 6M guanidinium chloride containing 10mM EDTA, 5mM NEM and 1mM PMSF pH 6.5. After mucus purification by caesium chloride density gradient ultracentrifugation, glycoprotein identification was performed by Western blotting and staining with PAS. Samples which identified positive for glycoprotein were run on a 2D SDS-PAGE gel, stained with Coomassie Brilliant Blue then the spots of interest excised and run on a MALDI-TOF-TOF for identification.

Blood samples were collected from patients 3 days after the gastrectomy (n=14). Normal blood was collected from volunteers (n=15). The concentration of AGP was determined with a human ELISA kit using the provided protocol. The levels of MUC1, MUC4, MUC5AC and MUC6 in the plasma were determined using a sandwich ELISA. Gastric tissue blocks were retrieved from the archives of the Division of Anatomical Pathology, National Health Laboratory Service, Groote Schuur Hospital. The expression of AGP and mucins, MUC1, MUC1core, MUC2, MUC4, MUC5AC and MUC6 were determined in the gastric tissue by immunohistochemistry techniques.

Results: The PAS positive protein on both 1 and 2-D gels was identified as AGP. Levels of AGP in blood plasma were lower in cancer patients (336µg/ml) than that of healthy controls (547µg/ml) (*p*-value=0.00006). This was also observed in the adenocarcinoma tissue where the AGP expression was lower than normal gastric tissue. Cancer plasma levels were lower than controls for MUC1 (*p*=0.00017) and MUC5AC (*p*-value=0.02308). No difference was observed in MUC4 (*p*-value=0.343) and MUC6 (*p*-value=0.379) plasma levels between the two groups. Tissue staining of MUC1 and MUC4 did not correlate with ELISA findings but a correlation was observed with MUC5AC and MUC6. *De novo* MUC2 expression was observed in adenocarcinoma and intestinal metaplasia tissue.

Summary: The lower levels of AGP-MUC1 combination in the blood of cancer patients would have potential as a marker for early gastric disease.

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Thank you to my family and friends for their never-ending support and always reminding me that the end was in sight. Thank you for inspiring me through your words and strengthening my belief in that I can achieve what I set my mind to.

Lastly but most importantly, all praise and honour to God. Thank you for always guiding my footsteps, even when I was not aware.

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Abbreviations

A405	absorbance at 405nm
AMPS	ammonium persulphate
CHAPS	3-((3-cholamidopropyl)-dimethyl-ammonio))-1-propanesulfonate
CsCl	caesium chloride
DAB	3,3' -diaminobenzidine
DMSO	di-methylsuphoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
GuHCl	guanidinium chloride
HCC	hepatocellular carcinoma
H&E	haematoxylin and eosin
HID	high iron diamine
HPLC	high-performance liquid chromatography
HRPO	horse radish peroxidase
H_2O_2	hydrogen peroxide
H_2SO_4	sulphuric acid
IAA	iodoacetamide
IEF	isoelectric focusing
IPG	immobiline pH gradient
kDa	kilo-Dalton
mA	milliamper
MALDI-TOF	matrix assisted laser desorption ionization time of flight
MS	mass spectrometry
MUC	mucin

M**W**	molecular weight
NEM	N-ethlymaleimide
PAS	periodic acid Schiff
PAS/AB	periodic acid Schiff/alcian blue
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
rpm	revs per minutes
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide-gel electrophoresis
TEMED	N, N, N′, N′ – Tetramethylethylenediamine
TMB	3,3′,5,5′-Tetramethylbenzidine
Tween 20	polyoxyethylene sorbitan monolaurate
VNTR	variable number of tandem repeats
V	voltage

Chapter one - Introduction

Background

There has been an increasing interest in mucins in the detection and treatment of carcinomas in general (Devine et al. 1992) and also in particular in their role as diagnostic and therapeutic agents (Graham et al. 1996). Tumour markers useful in diagnosing gastric carcinoma at an early stage have not been identified. Many of them, such as carcinoembryonic antigen are most often elevated in patients with incurable disease and are therefore not useful for early detection (Fuchs et al. 1995). Hakkinen et al (Häkkinen et al. 1991) described a structure associated with gastric mucins in carcinoma that could be useful as a clinical marker, and Bara et al (Bara et al. 1988) showed that ovarian mucinous cysts contain common antigens with the mucous positive columnar epithelial cells of the normal gastric mucosa. Our lab has reported the fragmentation of MUC5AC, an epithelial gastric mucin that consistently generated a 55-65kDa glycoprotein in crude mucus scrapings and some gastric juice specimens from the stomachs of patients with ulceration, carcinoma or Menetrier's disease. The data suggested this glycoprotein to be the response of the gastric mucosa to early injury in the form of inflammation, gastritis and intestinal metaplasia (Mall et al. 1999; Mall et al. 2000; Mall et al. 2002). Other likely roles for this fragment suggested by mucinologists overseas were that it could act as an attachment factor for *H. pylori* (J Dekker, Amsterdam, personal communication) or a precursor of human gastric mucin (Klomp et al. 1994). Another possibility was that because of its N-linked (as opposed to its O-linked) glycosylation (Mall et al. 1999) , it could be similar to the MUC1 epitopes produced by mammary tumour cells, which acquire an N-linked glycosylation after normal cells become transformed (Hilkens et al. 1988). Previous studies from our laboratory (Mall et al. 1990; Mall et al. 1992) showed the 55-65kDa fragment fractionated with albumin, raising the question of the incomplete purification of mucin from crude mucus secretions. However, albumin 'contamination' has more recently been experienced by other researchers (I Carlstedt, University of Lund, Sweden, personal communication and in plenary session of ICRF Mucus in Health and Disease Workshop, Cambridge UK, 2000).

Nthato Chirwa raised a polyclonal antibody to AGP for his PhD (2008) entitled "The isolation, purification, tissue localization and identification of a glycoprotein found in the crude mucus gel of patients with carcinoma of the stomach" and published paper "A 40-

50kDa glycoprotein associated with mucus is identified as α-1-acid glycoprotein in carcinoma of the stomach" (Chirwa et al. 2012). Using immunohistochemistry, Chirwa et al. (2012) showed that it was expressed in the parietal cells of the normal stomach and enterocytes of the small intestine. It was more highly expressed in diseases such as intestinal metaplasia and chronic gastritis and its highest expression was in the gastric tumour, explaining why it could be detected in crude mucus scrapings of patients with carcinoma of the stomach. AGP preservation from normal tissue through to pre-neoplastic changes to the formation of tumour is interestingly different to that of mucins, which are known to be down-regulated in pre-malignancy. AGP stained for protein and carbohydrate on 2D gel-electrophoresis and whilst the protein was identified, our researcher was unable to identify the glycosylated component of the protein because it was resistant to digestion with trypsin. These findings raise the question of AGP having potential as a marker for pre-malignancy or even a diagnostic and/or prognostic marker for stage of disease. The protein was identified by MALDI-TOF MS as α -1 acid glycoprotein (oroscomucoid), an immune suppressant in certain cancers (Chirwa et al. 2012).

Gastric cancer is prevalent in the Western Cape region of South Africa and to date, there has not been a suitable biochemical marker for pre-malignant disease. New patients reporting to hospital present with pain and undergo endoscopy, followed by biopsy and histology to confirm diagnosis. In most instances, upon diagnosis of malignancy, it is too late to treat and surgery is performed to alleviate the condition. The five year survival figures for these patients are low. The development of a biochemical marker would be a significant contribution to the management and to the survival outlook of this disease. Thus our lab sought to determine whether α-1 acid glycoprotein will be a suitable clinical marker for early gastric disease.

1.1 Mucus

Mucus is a slimy thick substance secreted by cells located on epithelial surfaces throughout the gastrointestinal tract and acts as a medium for protection and lubrication of epithelial surfaces. It has antiprotease, antimicrobial and antioxidant properties which aid in its various functions (Voynow et al. 2009). It also prevents the auto-digestion of the stomach wall by hydrochloric acid which is produced by the parietal cells and helps moisten food as it travels down the oesophagus (Corfield et al. 2001). In the respiratory system, mucus acts as the first line of defence by trapping inhaled particles or pathogens (bacteria and viruses) and clears the respiratory mucosa via the action of microcilia (Thornton et al. 1997; Thornton et al. 2004). Mucus can also be found in the cervix where it protects the cervical epithelium from desiccation as well as regulating the activity of spermatozoa during the menstrual cycle (Carlstedt et al. 1983a).

1.12 Mucins

Mucus is predominately made up of water and mucins which are large (Mr 1×10^6 - 50×10^6) heavily O-glycosylated glycoproteins. The high level of glycosylation gives mucins their gel-forming property (Jentoft 1990). There are two main types of mucin: monomeric mucins which are mostly located on the cell surface and oligomeric mucins which are secreted. Mucins have peptide sequences which contain tandem repeat regions. These sequences are enriched with serine, threonine and proline residues, known as PTS regions, and do not normally have cysteine residues. These PTS regions are normally long (up to 6000 amino acids) and usually make up more than half of the polypeptide (Dekker et al. 2002).

Mucin molecules contain up to 80% carbohydrate in the form of acidic or neutral oligosaccharide units arranged linearly and in branched sequences (known as the bottle-brush model) (Thornton et al. 2004). The sugars are attached to the protein core (apoprotein) through oxygen linkages (O-linked glycosidic bonds). The oligosaccharide chain on a mucin consists of: *N*-acetylglucosamine, *GalNAc*, galactose, fucose and sialic acid where *GalNAc* normally attaches to the apoprotein via an O-linked glycosidic bond. Each of the branched chains has varying numbers of sugar residues where the terminal sugars are usually sialic acid, fucose, galactose esters and *N*-acetylglucosamine. The highly glycosylated regions are densely packed into globular structures and are interspaced with less glycosylated regions of

protein that are vulnerable to proteolytic degradation to form glycopeptides. The less glycosylated regions do not contain tandem repeats and are rich in charged amino acids. The N- and C-terminals of the polypeptides are non-PTS regions and usually encode for all the cysteine residues present in mucins (Dekker et al. 2002; Peppas et al. 2004).

One mucin molecule has four subunits and each subunit is made up of a basic unit approximately 5×10^5 in weight, consisting of one branched chain and one or two terminal peptide sequences. Individual mucin peptides are connected by intramolecular disulphide bonds which enables the reduction of mucins to form smaller subunits using reducing agents (Strous et al. 1992; MacAdam 1993; Gandhi et al. 1994; Fiebrig et al. 1995; Peppas et al. 2004; Rose et al. 2006). Mucin molecules are joined together by intermolecular interactions that include hydrogen bonding, physical entanglements and hydrophobic interactions to form macromolecules (Creeth 1978; Sheehan et al. 1990; MacAdam 1993; Peppas et al. 2004). Figure 1.1 below shows a diagram of one mucin polypeptide.

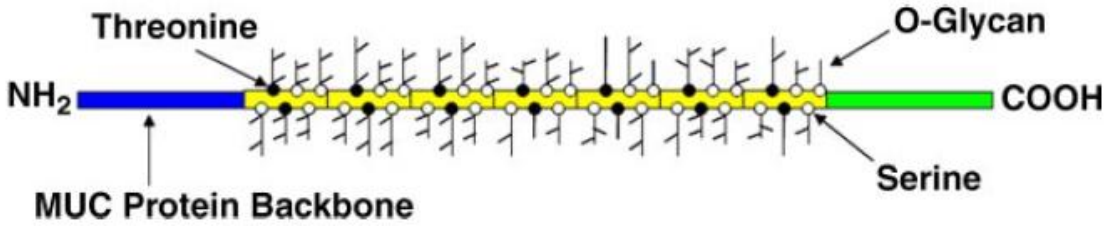


Figure 1.1: Schematic drawing showing the apoprotein with its tandem repeats (○) and O-glycans attached to serine (○) or threonine (●) residues. The N-terminal is indicated by the blue and C-terminal indicated by the green where the non-PTS regions are located. Reproduced from (Rose et al. 2006).

1.13 Classification of mucins

Mucin genes code for mucins according to their structure that can either be monomeric which produce membrane-bound mucins, or they can be oligomeric which form secreted or gel-forming mucins. Table 1.1 shows all the MUC genes and their chromosomal location.

MUC Gene	Chromosome Locus
MUC1	1q21-q24, 1q21
MUC9	1p13
MUC13	3q13.3
MUC4	3q29
MUC20	3q29
MUC7	4q13.3
MUC3A	7q22
MUC3B	7q22
MUC11	7q22
MUC12	7q22
MUC17	7q22
MUC2	11p15.5
MUC5AC	11p15.5
MUC5B	11p15.5
MUC6	11p15.5
MUC15	11p14.3
MUC18	11q23.3
MUC19	12q12
MUC8	12q24.3
MUC16	19q13.2

Table 1.1: Chromosomal loci of human mucin genes as listed in GenBank. Reproduced from (Rose et al. 2006).

1.13.1 Membrane-bound mucins

MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC16, MUC17 and MUC20 are membrane bound mucins (Rose et al. 2006). They usually have a transmembrane domain, a sea-urchin-sperm-protein-enterokinase-agrin (SEA) domain and an epidermal-growth-factor (EGF) domain. MUC3A, MUC3B and MUC12 are closely related as they are located on chromosomal locus 7q22 (Dekker et al. 2002). The main function of membrane-bound mucins is to communicate extracellular information via intracellular signal transduction pathways. All membrane-bound mucins prevent cell surface proteolysis in addition to attacks from other cells. The mucin genes have cell or tissue specific expression but one tissue can express different types of mucins (Bansil et al. 2006; Rose et al. 2006).

1.13.2 Secreted (gel-forming) mucins

The secreted mucins include MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC9 and MUC19. Secretory mucins are split up into two classes, gel-forming polymeric mucins and non-gel forming soluble mucins.

The secreted and gel-forming polymeric mucins include MUC2, MUC5AC, MUC5B, MUC6 and MUC19 and are all found at the chromosomal locus 11p15.5. These five mucins have homologous non-PTS regions and have von-Willebrand-factor like (vWF) domains and a conserved C-terminal domain (Dekker et al. 2002). The gel forming properties are linked to the cysteine rich domains within the C- and N-terminals that form polymers through disulphide bridges. These disulphide bridges are critical for mucus gel formation.

The secreted and non-gel forming soluble mucins only include MUC7, MUC8 and MUC9. MUC7 is small but unlike the other secretory mucins, it does not contain cysteine-rich domains therefore does not form gels. MUC8 is a large mucin that does not have the vWF as well as the C1 and C2 domains on the C-terminal end of large mucins. All the secreted forms have a role in transport and also interact with membrane bound mucins through covalent and non-covalent bonds (Rose et al. 2006).

1.14 Synthesis of Mucins

1.14.1 Overall mucin synthesis

Mucin biosynthesis takes between 6-24 hours. The process begins firstly by transcription of the MUC gene in the nucleus and thereafter the transcript is transported to the endoplasmic reticulum where translation occurs. The MUC protein (apoprotein) is the first to be synthesised and then intramolecular disulphide bond formation occurs as well as N-glycosylation. The start of O-glycosylation occurs in the cis-Golgi body and then the N-acetylgalactosaminyl peptidyltransferase enzyme facilitates the transfer of N-acetylgalactosamine (GalNAc) to the threonine or serine amino acids. The transfer of hexoses and hexosamines then occurs to each emerging O-glycan in a stepwise process by different glycosyltransferase enzymes (Kirnarsky et al. 1998). After post-translational modification, mucins are either stored in vesicles (granules) to be secreted (secretory mucins) or incorporated into the membrane (membrane-bound mucins) (Corfield et al. 2001; Rose et al. 2006).

1.14.2 O-glycans structures

There are a number of core types of O-glycans that are attached to the apoprotein during mucin synthesis which make up a large part of the mucin molecule. These include GalNAc, N-acetylglucosamine, N-acetylneuraminic acid, fucose and galactose. Figure 1.2 shows the major core structures of O-glycans specifically found in mucins. Terminal sugars found on the O-glycans are important as they determine the biological and/or physical properties of mucins by their charge or hydrophobicity. Mucin terminal sugars are often altered in disease and this can alter their function or physical property (Rose et al. 2006).

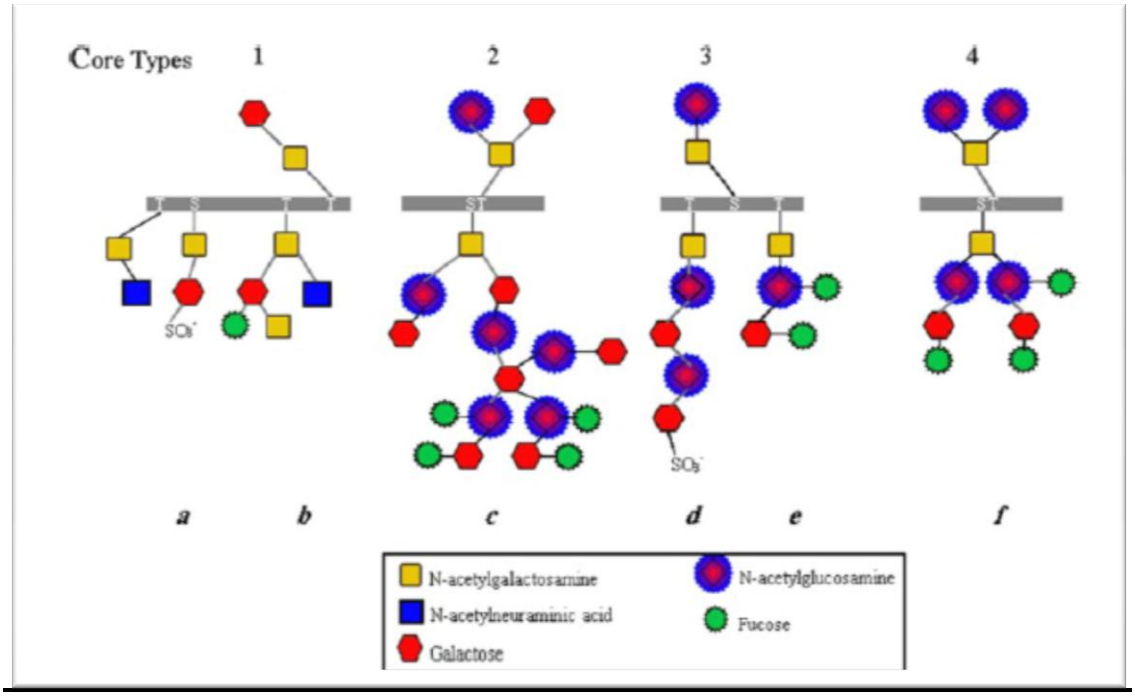


Figure 1.2: The core structures of the O-glycans which surround the apoprotein ( ). Each core structure is built upon with either fucose, GalNAc, N-acetylglucosamine, N-acetylneuraminic acid or galactose. Reproduced from (Rose et al. 2006).

1.15 Biochemical Structure of Mucin

Mucin molecules have always been difficult to study due to their large bulky structure, heavily glycosylated regions and the dissimilarity between seemingly related mucins. Early mucus studies proposed two possible structures for the mucin subunits (shown below in Figure 1.3); the windmill or star form by Allen et. al (Allen et al. 1972) (Figure 1.3a), then the linear form by Carlstedt and Sheehan (Carlstedt et al. 1984) (Figure 1.3b). The windmill model was first supported by the susceptibility of mucin to the attack by thiols and proteases. Thereafter estimates of the native and reduced molecular weights of pig gastric mucin suggested the presence of four subunits (Allen et al. 1972). Analysis of amino acid end groups and the discovery of a 70,000 M_r linker protein also supported the windmill model for mucins (Pearson et al. 1981). Carlstedt and Sheehan's results contradicted Allen's results as they obtained mucin forms of much larger molecular weight found with pig gastric mucin (Carlstedt et al. 1984). Further support for the linear form was shown when nodule-like forms in linear chains were observed using electron microscopy. These chains were of varying lengths and more importantly, no branched forms were observed (Mantle et al. 1981; Carlstedt et al. 1983b; Sheehan et al. 1986; Peppas et al. 2004). Further work soon showed that the linear form was correct whereby mucin molecules formed flexible strands or subunits connected by disulphide bonds that ultimately formed an overall globular structure (Carlstedt et al. 1985; Sheehan et al. 1991; Thornton et al. 1997).

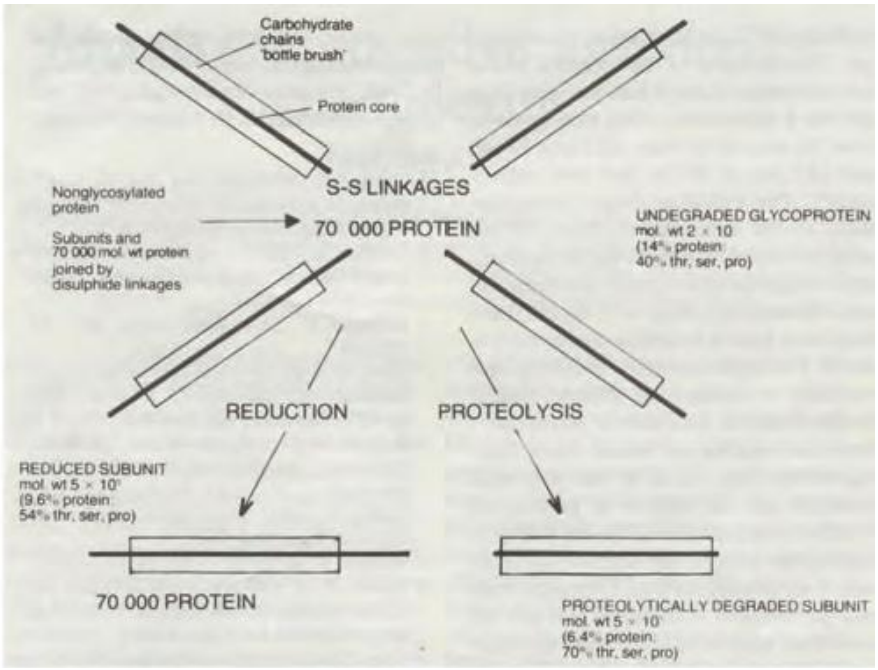


Figure 1.3a: Proposed structure by Allen et. al for 'Windmill' model of mucin subunits. Four subunits linked by a linker protein. Reproduced from (Allen et al. 1972).

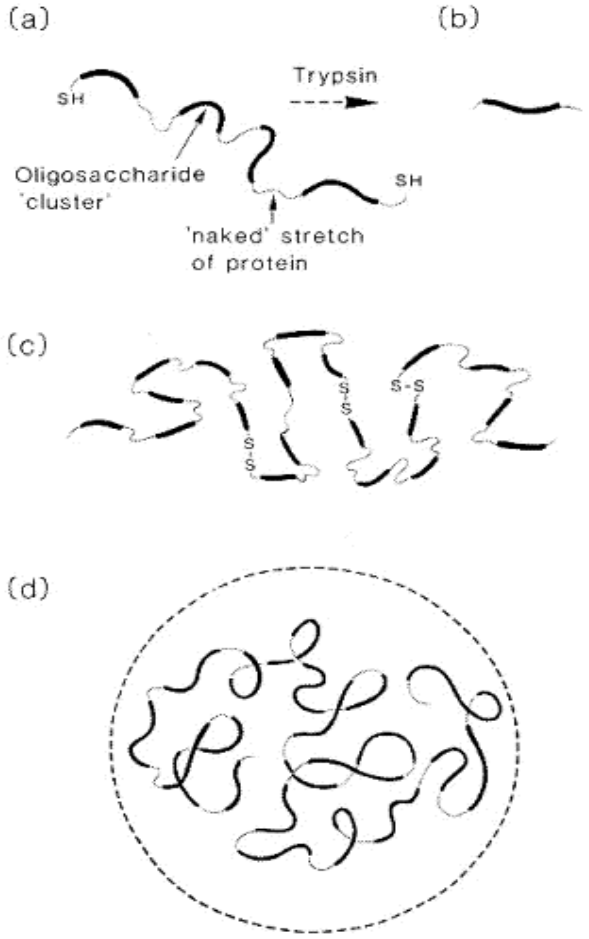


Figure 1.3b: Schematic of proposed structure by Carlstedt and Sheehan for linear flexible model of mucin glycoprotein. Subunits linked end to end via disulphide bridges. Reproduced from (Carlstedt et al. 1984).

1.16 Mucin purification, proteolysis and reduction

As previously stated, mucus is a complex macromolecule and thus it is difficult to isolate, reduce and break down to its smaller subunits. Mucins are isolated by treatment with 6M guanidinium chloride (GuHCl) and protease inhibitors to protect the primary structure from degradation. Thereafter the mucins are purified by two rounds of caesium chloride (CsCl) isopycnic density gradient ultracentrifugation. The density for this purification is fixed at between 1.39 and 1.40g/ml. Proteolytic degradation occurs when enzymes target the accessible non-glycosylated regions of the protein core and form mucin monomers known as 'T-domains'. These monomers are end-to-end subunits connected and stabilised by folded intramolecular disulphide bonds (shown in Figure 1.4A). On the other hand when mucus is reduced with reducing agents such as dithiothreitol (1, 4-dithio-d-threitol) and 2-mercaptoethanol (β-mercaptoethanol), approximately 600 nm length subunits form (shown in Figure 1.4B). The reduced subunits have unfolded disulphide bonds at each end (-SH bonds) and are referred to as 'subunits'(Mantle et al. 1981). Figure 1.4 shows electron micrographs of the two structures that form after proteolysis and reduction.

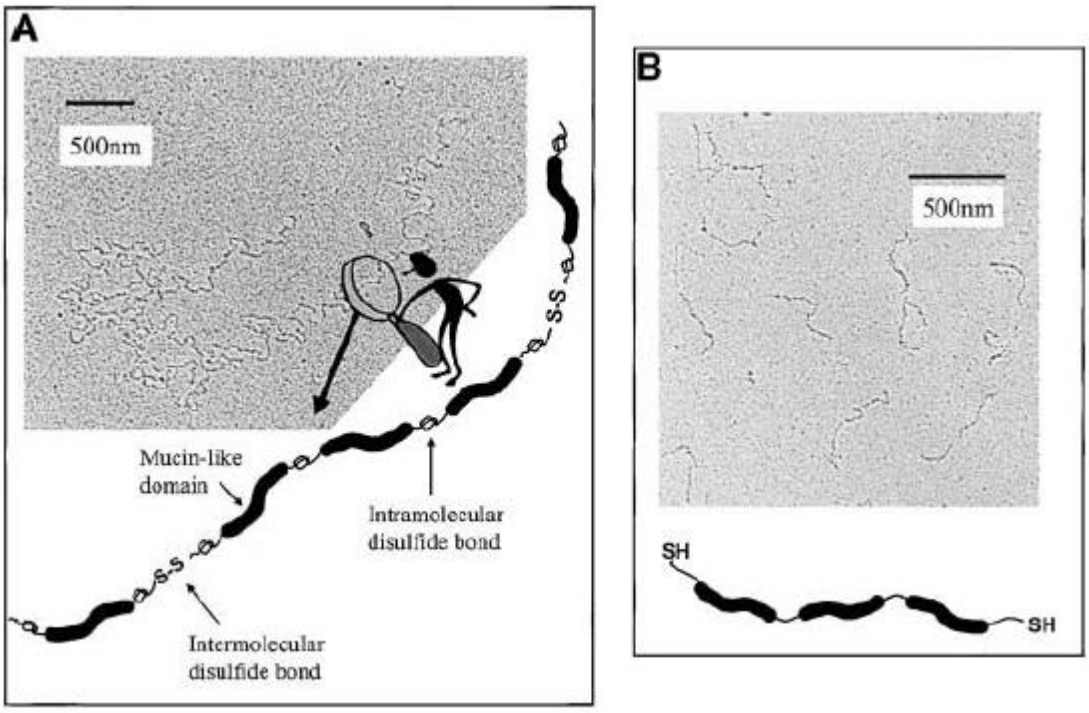


Figure 1.4: A: End to end disulphide bonds form between the subunits. The formation of T-domains occurs after proteolytic attack of the non-glycosylated regions **B:** Subunits which are the products of reduction and form short strands with –SH bonds at each end. Reproduced from (Thornton et al. 2004).

1.2 Gastric mucus and mucins

1.21 Gastric mucus

Figure 1.5a shows a schematic of the full stomach anatomy. Figure 1.5b shows a section from the wall of the stomach that has been magnified. This shows that the wall of the stomach has five layers which include from the lumen, the mucosa then the submucosa, the underlying muscle layer called the muscularis then the subserosa and the serosa. There is an upper loose layer of mucus which acts as a lubricant wall from the mechanical stresses that occur during digestion. The lower layer of mucus within the stomach forms a stable continual insoluble gel layer which has strong adhesion properties and thus adheres to the luminal surface of the gastric epithelium. Gastric mucus consists mainly of water then mucins, electrolytes, enzymes and other components such as bacteria and nucleic acids (Hotta 2000). Figure 1.5c shows the undisturbed layer of gastric mucus which allows for the retention of bicarbonate ions (HCO^{3-}) secreted by the epithelial cells that neutralise some of the hydrochloric acid (HCl) and protects against pepsin proteolysis. The HCO^{3-} secretion is stimulated by the presence of acid in the stomach. The unstirred mucosal gel layer allows for a gradient to be formed from the luminal surface (acidic pH 1-2) to the epithelial surface (near neutral pH 7) so as to prevent damage to the epithelial cells shown in Figure 1.5d (Allen et al. 1979; Allen et al. 1984; Ichikawa et al. 2011). The overall efficiency of gastric protection by mucus is established by the thickness of the mucus layer which is determined by hormones, neural and paracrine stimulation (Taylor et al. 2000; Allen et al. 2005).

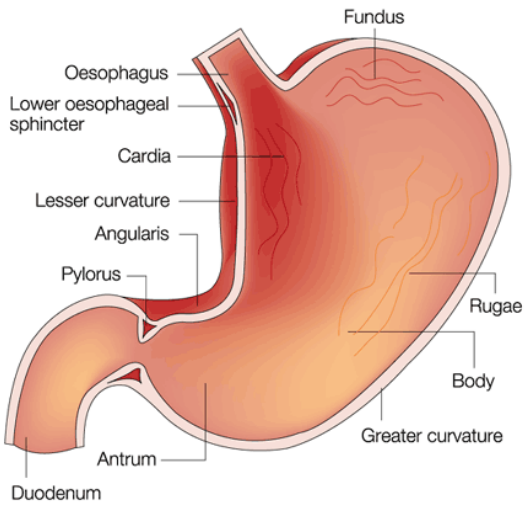


Figure 1.5a: Full cross section schematic showing the areas and structures of the anatomy of the stomach. Reproduced from (Peek et al. 2002).

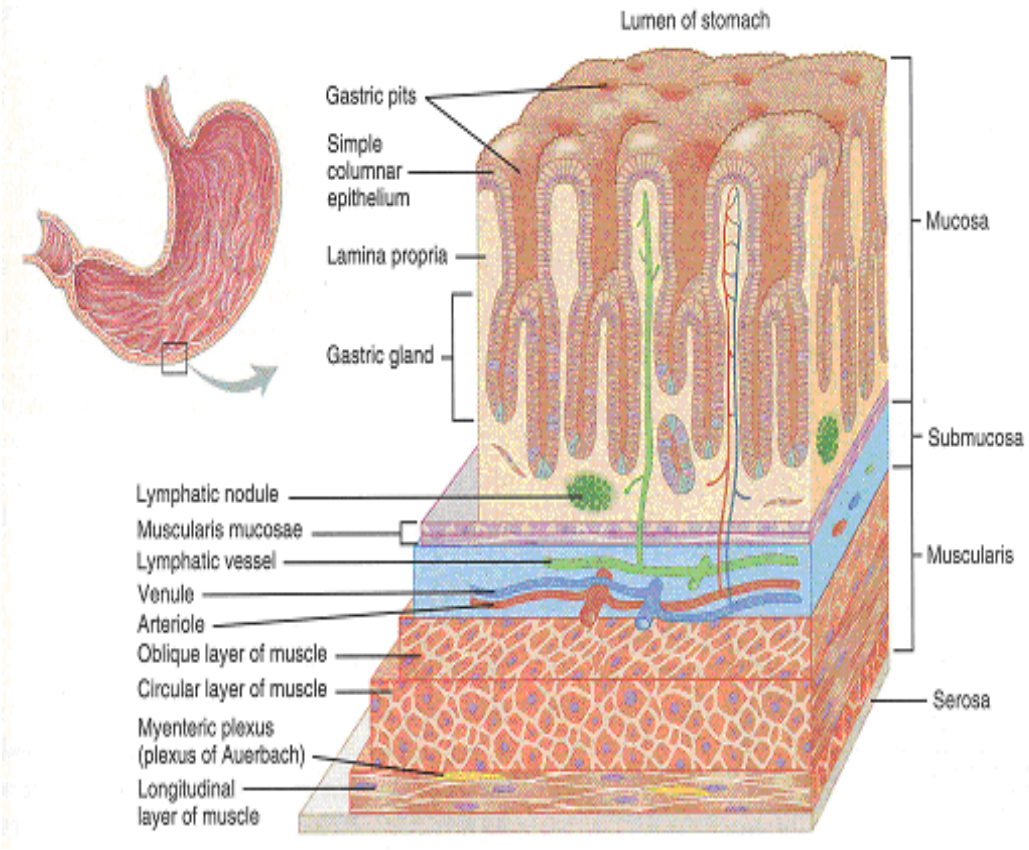


Figure 1.5b: Schematic of layer of the inner gastric anatomy(edoctoronline.com).

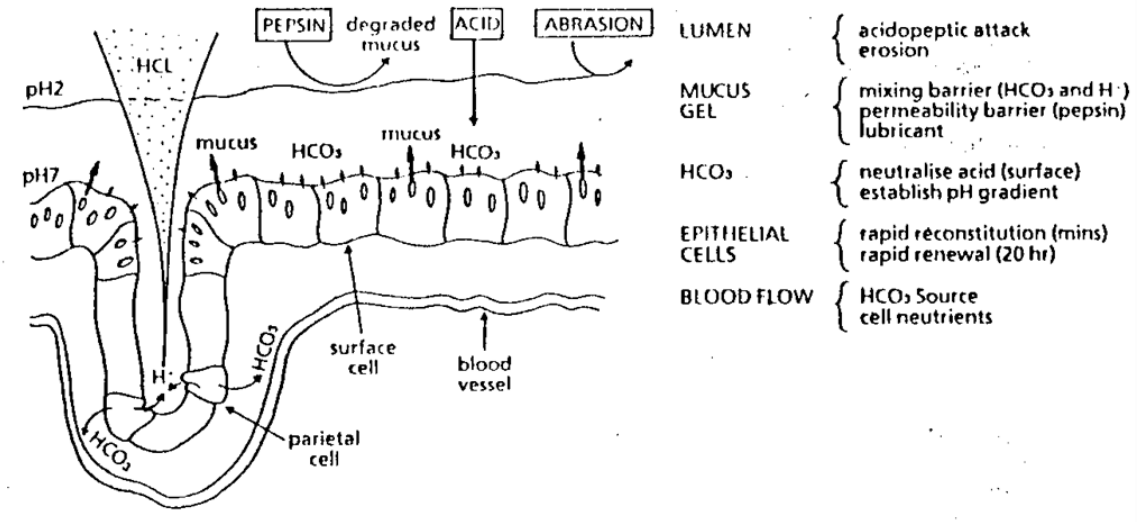


Figure 1.5c: A schematic showing the distribution of cells and gel mucus layer which allows for the neutralisation of stomach acid and pepsin by HCO^{3-} retention. Reproduced from (Allen et al. 1984).

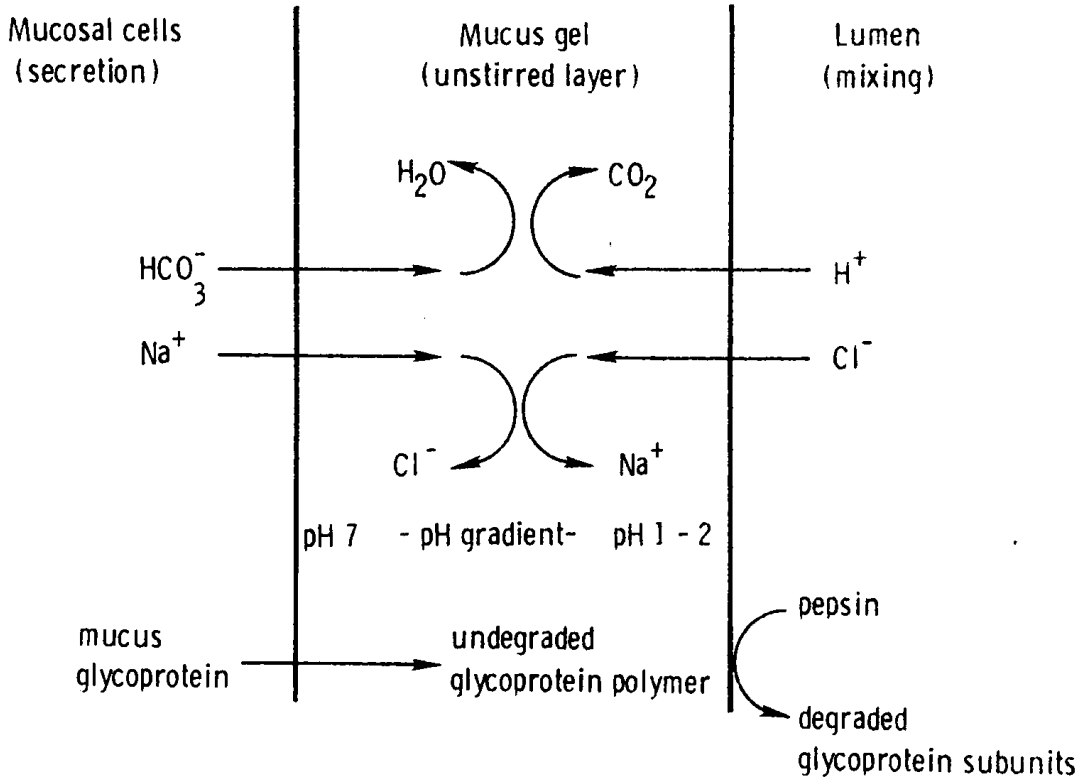


Figure 1.5d: Unstirred mucus layer which forms a gradient from the lumen (pH 1-2) to the epithelial cells (pH 7) which allows HCl neutralisation by HCO^{3-} to prevent gastric epithelial cells damage. Reproduced from (Allen et al. 1979).

The gastrointestinal tract has a mucus layer that protects against infection. In the stomach and large intestine there is a two-layered mucus gel that protects against infection and inflammation. This includes the inner stratified mucus layer and the outer mucus layer. The inner layer is 50-200µm in thickness and provides physical protection through the glycocalyx of the epithelial cells. The outer layer is less defined and is easily removed (Johansson et al. 2011; Hansson 2012).

Gastric mucus also acts to protect the lining of the stomach after injury to prevent further mucosal damage. Figure 1.6 shows the difference in the mucus layer thickness between the undamaged (Figure 1.6A), ethanol damaged (Figure 1.6B) and ethanol and saline damaged (Figure 1.6C) in a rat model. This work showed that after gastric epithelium was exposed to 70% (v/v) ethanol and then to a saline solution, a mucus layer formed over the damaged epithelial cells. This mucosal layer was ten times thicker than the undamaged saline treated control group of gastric epithelial cells. (Sellers et al. 1987). Similar results are observed in human gastric tissue where the mucus gel of ulcerated tissue was five times thicker than that of the normal tissue (Allen et al. 1990).

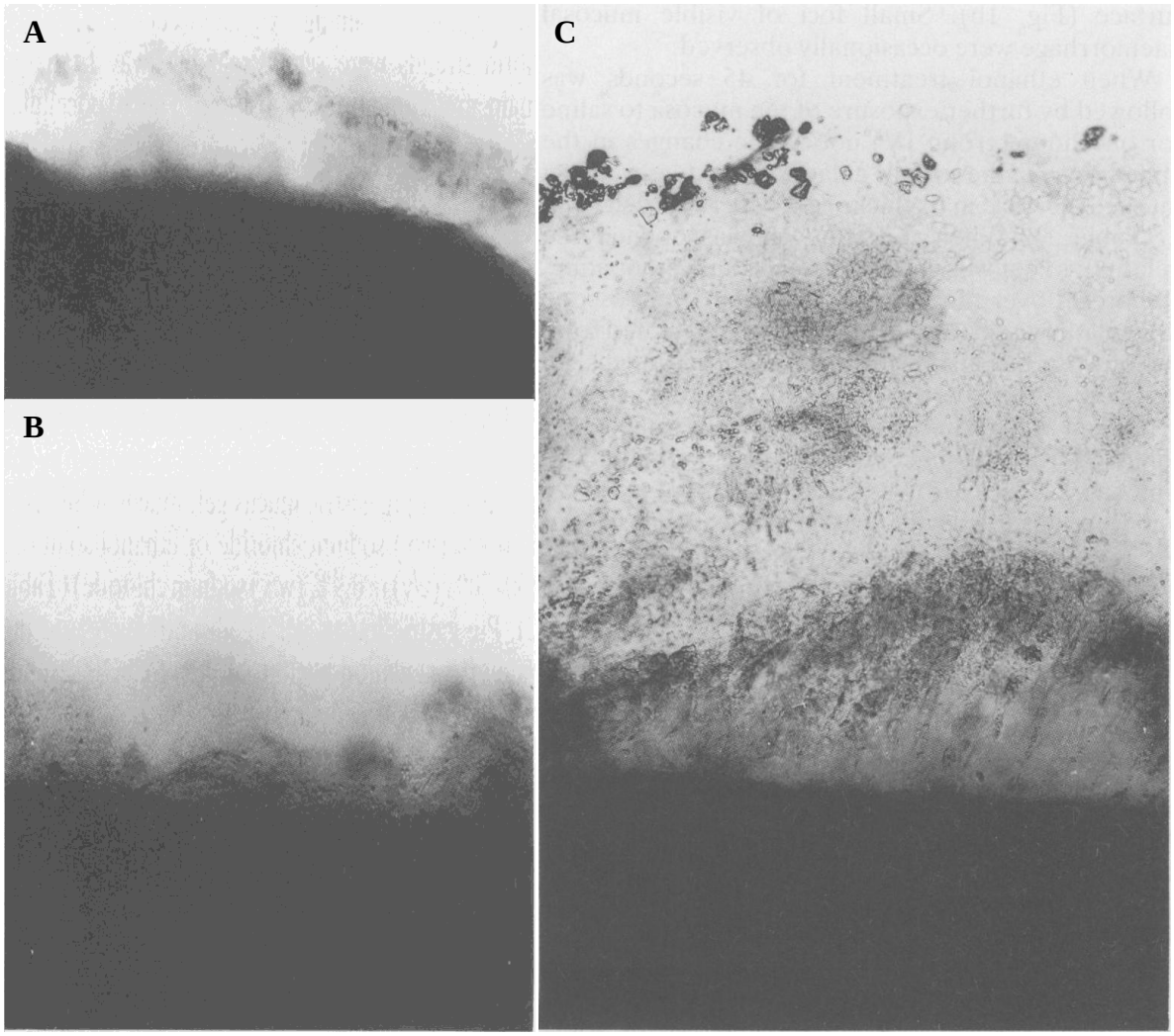


Figure 1.6: A: Rat mucosa of saline control group (undamaged), average mucosa thickness was 70µm **B:** Ethanol exposed (damaged) mucosa layer, average of 20µm. **C:** Re-epithelialised mucosa exposed to ethanol then to saline solution was much thicker than the normal mucosa, average mucosa thickness of 680µm. Reproduced from (Sellers et al. 1987).

1.22 Gastric mucins

There are four mucins found within the gastric regions namely, MUC1, MUC4, MUC5AC and MUC6. There are a number of components which influence the behaviour of these mucins in the stomach such as the trefoil factors family (Sands et al. 1996; Thim et al. 2002), *Helicobacter pylori* (*H.pylori*) infection (Lindén et al. 2004), tumour-associated antigen (Corfield et al. 2000) and mucin terminal glycans (Corfield et al. 2001). These will b e discussed in a later chapter.

MUC1 is a membrane-bound mucin located on chromosome 1q21-24. It has a cytoplasmic tail and extracellular domain. The mucin is located on the cell surface of epithelial cells and aids in protection by binding pathogens as well as functioning in intercellular cell signalling pathways. In solid tumours, a MUC1 variant is normally formed whereby the O-glycans are shortened and less branched (Taylor-Papadimitriou et al. 1999) .

MUC4 is located on chromosome 3q29 and is expressed in the epithelium of the digestive tract. It provides protection and lubrication to epithelial surfaces. MUC4 consists of two sub-units, the extracellular glycosylated MUC4-a and the transmembrane subunit with epidermal-growth factor-like domains, MUC4-b. The extracellular subunit may have specific adhesive functions which could aid in metastasis when MUC4 expression changes in cancer, whereas the transmembrane subunit acts in cell signalling pathways. MUC4 has been implicated in pancreatic, lung and colon cancer but studies have not conclusively shown a change in expression in gastric cancer (Dekker et al. 2002).

The MUC5AC gene is found on chromosome 11p15.5 and is highly expressed in gastric mucosa. MUC5AC, together with MUC6, are both key constituents of the protective layer covering the gastric epithelium, thus facilitating the formation of a pH gradient from the luminal surface to the epithelial surface as described earlier in chapter 1.12. It also protects against *H.pylori* as the O-glycans act as ligands for the bacteria.

MUC6 is highly expressed in the gastric mucosa. It is located on chromosome 11p15.5 and it is about 24kb in length. MUC6 is located in the deep gland cells and is produced by the acinar cells of the duodenal Brüner's glands. The glycans located on MUC6 have antimicrobial activity which aid in combating *H.pylori*. Some studies have found that shortened alleles of MUC6 are associated in *H.pylori* infection and gastric cancer (Corfield et al. 2000; Bansil et al. 2006).

1.23 Gastric mucin and *H.pylori*

The stomach has a very hostile acidic environment in which most bacteria are unable to survive. Despite this, *H.pylori* is found in the stomach of 50% of the human population though the majority of them are asymptomatic. Infection with this bacterium is related to the development of gastric ulcers, gastritis and gastric adenocarcinoma and increases the risk of developing cancer by almost 3 times the normal amount.

Mucins have the ability to bind bacteria by specific attachment sites through the presence of receptor analogue structures. Figure 1.7 shows the interaction between the bacteria and the gastric mucins. MUC5AC and MUC6 expression changes when *H.pylori* is present in the stomach. The MUC5AC receptor competes with the bacteria for binding sites on the epithelial surface. MUC6 carries a terminal α-1-4 linked N-acetylglucosamine residue that acts as an antibiotic against the bacteria (Zhang et al. 2001; Kawakubo et al. 2004). *H.pylori* causes a reduction in the renewal of the mucus layer but treatment of the infection results in the reversal of symptoms and the stomach goes back to its normal state (Corfield et al. 2000; Dekker et al. 2002; Lindén et al. 2004).

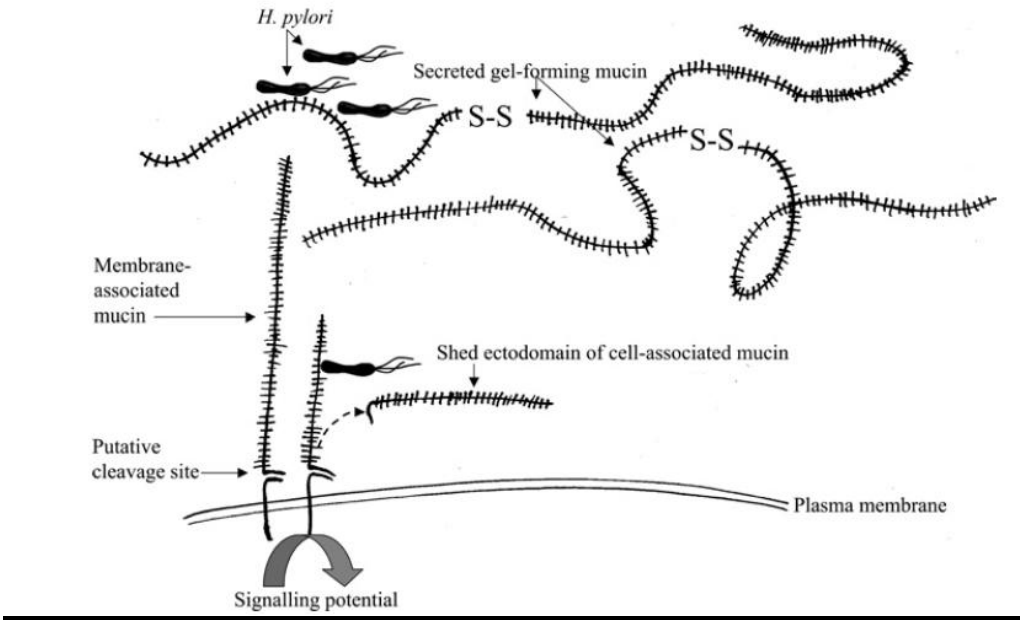


Figure 1.7: Interaction between *H. pylori* and secreted MUC5AC and MUC6 in the stomach. Reproduced from (Lindén et al. 2004).

1.24 Tumour-associated antigens

When the gastric tissue is infected or diseased, for example, when it transforms to neoplastic tissue, mucin production is altered. Glycosylation of mucins is usually affected resulting in the expression of tumour-associated carbohydrate antigens which contribute to the pathogenesis of disease (Corfield et al. 2000) . These are made up of mucin-type carbohydrate antigens such as Tn, sialyl-Tn and T epitopes that form as a result of incomplete glycosylation which leads to production of short O-glycans shown in Figure 1.8 (Corfield et al. 2000).

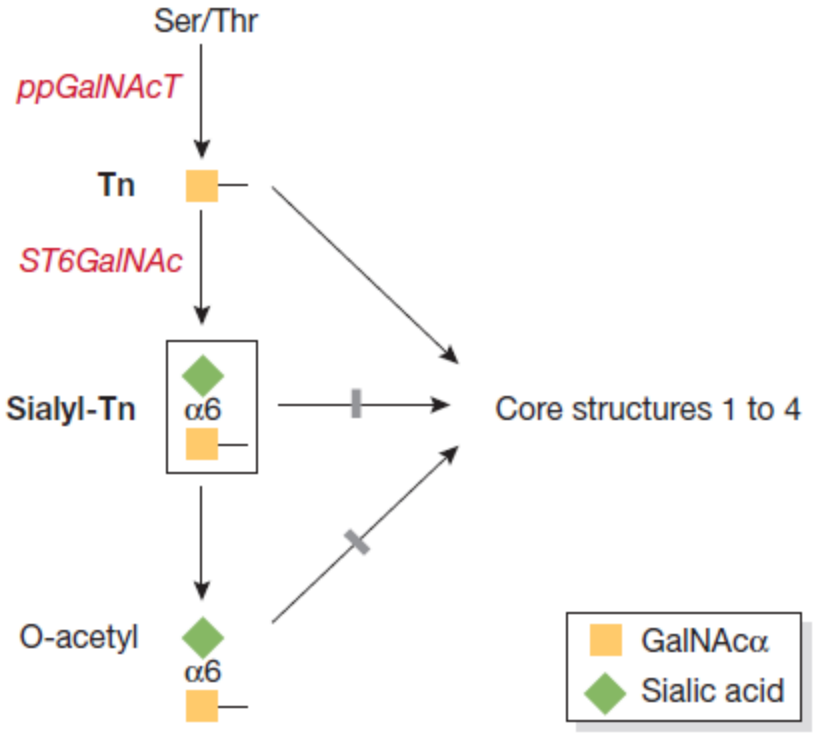


Figure 1.8: The shortened oligosaccharides are shown where Tn is linked to GalNAcα via a Ser/Thr bond and becomes sialyl-Tn when sialic acid is added. The T antigen is not shown above and would have a galactose linking to GalNAcα. In a normal environment of biosynthesis these O-glycans would be converted to the core structures and be heavily glycosylated (Singhal et al. 1990).

1.25 Mucin terminal glycans

Mucin terminal glycans are comprised of structures known as Lewis-type blood group antigens. These antigens are structurally related to determinants of the ABO blood group system and are highly expressed in normal gastric mucosa. Lewis^{x} and Lewis^{y} are found in the deep glands together with MUC6 and are also present in the chief and parietal cells in the stomach. Lewis^{a}, sialyl-Lewis^{a} and Lewis^{b} are co-localised with MUC5AC in the superficial cells. The over expression of these antigens is common in gastric, lung, pancreatic and colon cancer and is linked to poor patient survival and aggressive tumour metastasis (Corfield et al. 2001).

1.3 Mucus and mucins in disease

1.31 Mucus in disease and previous work

Mucus plays a key role in protection of the gastrointestinal tract and changes in mucin expression are seen in a number of diseases such as intestinal metaplasia, gastric ulceration, *H.pylori* infection and inflammatory bowel disease. Some work has been done in our laboratory to evaluate mucin expression in gastric diseases such as Menetrier's disease (Mall et al. 2003) and *Pseudomyxoma peritonei* (Mall et al. 2007; Mall et al. 2011) as mentioned earlier. Biochemical characterisation of gastric mucins from gastric cancer patients and patients with gastric ulcers showed that mucins from diseased stomachs were degraded. Gastric juices from these patients also showed a secreted 55-65 kDa glycoprotein with an unknown function (Mall et al. 1999). Important work has been done in our laboratory using a pig model investigating the effect of *H. helimannii* on mucosal changes using bile duct ligation. There was no association observed between infection with *H. helimannii* and the degradation of mucin (Mall et al. 2004). Further work has been done, again using the pig model with bile duct ligation to investigate changes in mucins caused by gastric ulceration. Purified mucins from pre-ulcerated and ulcerated stomachs had a degraded glycoprotein to total protein ratio that was higher as compared to normal stomachs. It was also found that purified mucin, GalNAc and fucose were decreased in the diseased stomachs (Mall et al. 1997). The foundation of this project was laid when the unknown 55-65 kDa glycoprotein which consistently co-fractionated with mucins from gastric cancer and ulcerated stomachs was found to be N-linked using HPLC analysis (Mall et al. 1999). After this work the unknown glycoprotein was identified as alpha-1-acid glycoprotein (AGP) or orosomucoid and its expression in gastric tissue was determined using immunohistochemistry, the results of which are shown and summarised in Figure 1.9 (Chirwa et al. 2012). After observing the different levels of expression of AGP in the gastric tissue, this project sought to determine the levels of AGP in blood plasma from gastric cancer patients and normal controls and determine if AGP may be a suitable biomarker for gastric cancer.

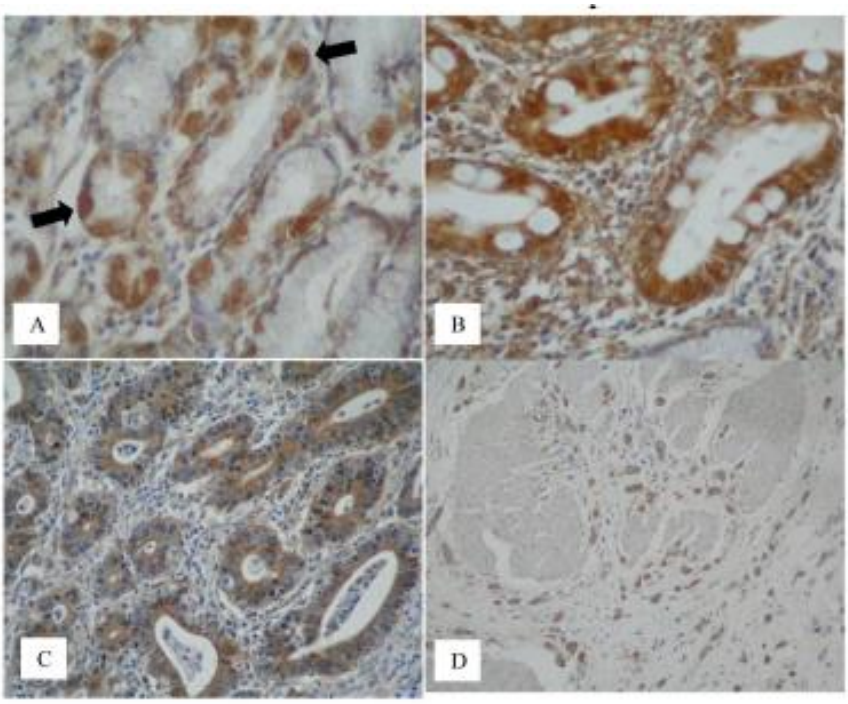


Figure 1.9: Sections of gastric tissue from normal, intestinal metaplasia and gastric cancer tissue stained with an antibody against alpha-1-acid glycoprotein which had been identified as the unknown protein of interest. **A:** Normal tissue (x40) showing dark staining therefore high expression in the parietal cells (indicated by the black arrows). **B:** Intestinal metaplasia (x40) showed high expression within the cytoplasm of columnar cells. **C:** Intestinal type carcinoma (x40) showed expression in the cytoplasm of the neoplastic glands. **D:** Diffuse type carcinoma (x20) showed expression in the cytoplasm of the neoplastic cells invading the stomach muscle wall. Reproduced from (Chirwa et al. 2012).

1.32 Gastric Cancer

In 2008, 12.7 million new cancer cases occurred worldwide with 7.8 million cancer deaths (Ferlay et al. 2010). A fair amount of these cases included gastric cancer (989,000 new cases in 2008) which is highly prevalent in the world and is the second leading cause of death as well as the fourth most common cancer in the world (Ferlay et al. 2010; Jemal et al. 2011). It is endemic in the Western Cape region of South Africa especially within the mixed (coloured) race population (Botha 1972; Bradshaw et al. 1985; Wyndham 1985). Gastric adenocarcinoma is the most common and makes up to 95% of gastric malignancies. Other types include primary gastric lymphoma which is the second most common, gastrointestinal lymphoma and carcinoids. Treatment methods include surgery, radiation and chemotherapy whereby the chance of survival decreases as the stage of the cancer increases (Lee et al. 2010).

The greatest risk factor for developing gastric adenocarcinoma is infection with *H.pylori* as it leads to inflammation and pre-neoplastic changes in the stomach wall. Other risk factors include stomach lymphoma, age (risk increases over 50), sex (more common in men), diet (risk increases with salty or smoked foods), tobacco use and obesity (Ferlay et al. 2010).

Sufferers of gastric cancer are often asymptomatic in the early stages of the disease and many of the symptoms are similar to those caused by other infections and diseases therefore early diagnosis of the disease is difficult. Symptoms include lack of appetite, stomach pain, weight loss, abdominal discomfort, nausea, vomiting, heartburn, indigestion and swelling of the abdomen.

Diagnosis is carried out by a number of tests with include upper endoscopy, upper gastrointestinal series, biopsy, endoscopic ultrasound, computed tomography, magnetic resonance imaging scans, positron emission tomography, chest X-rays, laparoscopy and laboratory tests which look for anaemia.

There is a need for a new cost effective diagnostic test to be developed for gastric cancer that could be used in hospitals and clinics as well as aid in screening methods. In Western countries the 5-year survival rate is very low whereas in Japan studies show survival can increase more than 50% due to the implementation of population based screening (Fukao et al. 1995). South Africa could benefit from a cost effective blood test that could be readily distributed and easily used.

1.3.2.1 Mucin Expression in Gastric cancer

Mucin expression has been shown to change as a result of gastric cancer. Normal MUC1 levels decrease in cancer as MUC1 tends to form abnormal glycoforms which are thought to aid in neoplastic transformation and metastasis through growth factor receptors and signalling molecule interaction (Bafna et al. 2010). De novo MUC2 and MUC3 expression occurs within the stomach with intestinal metaplasia. MUC4 expression in gastric adenocarcinomas is similar to MUC1 where aberrant glycosylation occurs as well as upregulation of the mucin (López-Ferrer et al. 2000; Senapati et al. 2008). MUC5AC expression is related to tumour stage in gastric cancer whereby it decreases as the tumour advances. In intestinal metaplasia, MUC6 expression decreases whereas incomplete metaplasia has no expression. Expression of MUC6 is lower in intestinal-type gastric carcinomas than in adenomas. This suggests it may aid in malignant transformation in the gastric epithelial cells (Corfield et al. 2000; Mall 2008).

1.4 AGP

Alpha-1-acid glycoprotein (AGP), also known as orosomucoid, is a 41-43kDa glycoprotein, a 183 amino acid protein that functions against inflammation and makes up 1-3% of plasma proteins (0.4-1 mg/ml) (Imre et al. 2008; Tesseromatis et al. 2011). It has 45% carbohydrate content connected via five to six highly sialylated complex-type-N-linked glycans. Figure 1.10, shows a schematic diagram of AGP which shows the branched sugar chains connected to the protein core. There are two to three main variants of AGP that are found in the blood plasma which are determined by the type of glycosylation and amino acid substitution that may occur (Fournier et al. 2000; Ryden 2002).

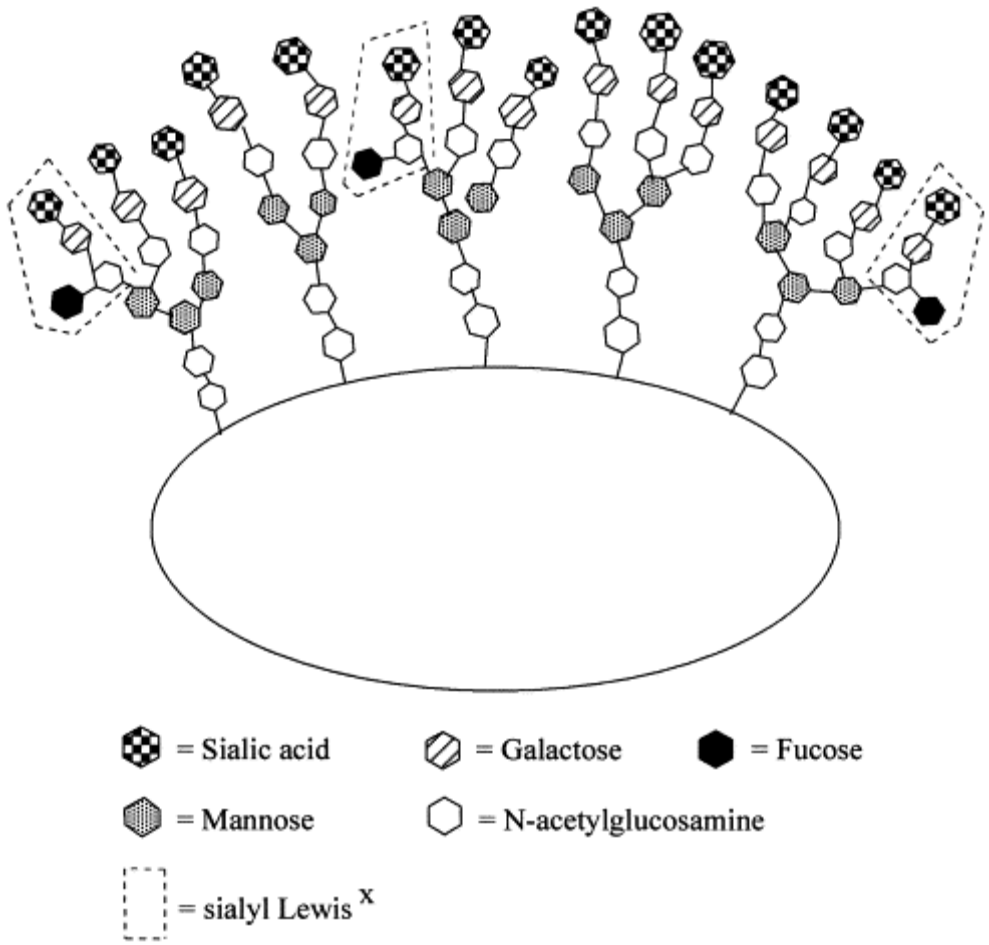


Figure 1.10: A schematic diagram of AGP showing the N-glycans attached to various carbohydrates in branched chains. These carbohydrates include sialic acid, galactose, fucose, mannose and N-acetylglucosamine. Sialyl Lewis^{x} shown by the dotted regions indicates a tumour antigen in gastric cancer. Reproduced from (Ryden 2002).

AGP is produced in the liver, secreted mostly by hepatocytes and is a member of the acute phase protein family. The plasma concentration of acute phase proteins increases greatly in response to systemic tissue injury, inflammation or infection (Fey et al. 1987). The increase in these proteins directly correlates with hepatic synthesis. AGP is largely involved in drug interactions (mainly basic drugs) by displacing drugs from their binding sites resulting in a pharmacokinetic response (Imre et al. 2008; Tesseromatis et al. 2011). Numerous studies have indicated that AGP may have diagnostic value. Plasma concentration of AGP is increased in breast, lung and ovarian cancer and it may have genetic variants that could be linked to each cancer. In hepatocellular carcinoma (HCC), levels of AGP have been shown to be higher compared to patients with chronic liver disease (Kim et al. 2006). AGP together with alpha-fetoprotein has a high sensitivity and accuracy in the diagnosis of HCC (Lee et al. 2006a; Bachtiar et al. 2009). It is because of this as well as the previous findings in our laboratory that we therefore postulate that gastric cancer patient prognosis could be improved by developing a test in which the ratios of AGP and various mucins in plasma are compared to ascertain an early diagnosis and which could also be used in population based screening.

Aim:

To investigate the potential of α-1-acid glycoprotein (AGP) as a biomarker for pre-malignant changes in the gastric mucosa and to investigate whether AGP and mucin (MUC1, MUC4, MUC5AC and MUC6) in blood plasma concentrations differ in cancer and healthy controls.

Objectives:

(1). To confirm previous results from our laboratory (Chirwa et al, 2012) where 3 blue spots and one pink spot were observed by Coomassie Blue and PAS staining respectively, on SDS-PAGE gels of mucins purified from crude gastric scrapings, by 2D gel electrophoresis.

(2). To identify the glycosylated component that stained for PAS on the gels from 2D gel electrophoresis by MALDI TOF analysis.

(3). To determine whether this marker (AGP) is present in blood by measuring its concentration using a commercial ELISA kit, comparing its levels in gastric cancer and healthy controls and relating its levels to stage of disease.

(4). To compare the expression of the trans-membrane (MUC1 and MUC4) and gel-forming (MUC5AC and MUC6) gastric mucins in cancer of the stomach and in healthy controls.

(5). To determine the expression of the abovementioned proteins including MUC1core and MUC2 in gastric tissue by immunohistochemistry.

Chapter two - Materials and Methods

2.1 Ethics

The University of Cape Town Research Ethics Committee re-approved this study on the 28th of June 2012 (HREC REF: 226/2009). Recruited patients were required to sign a consent form to indicate that they understood their involvement in the project.

2.2 Materials

Gastric mucus purification:

- GuHCl (Sigma-Aldrich)
- CsCl (Sigma-Aldrich)
- NEM (Sigma-Aldrich)
- PMSF (Sigma-Aldrich)
- EDTA (Merck)
- Dialysis tubing (Sigma-Aldrich)
- CHAPS (Sigma-Aldrich)
- Schiff reagent
- Period acid solution (Merck)
- Bradford assay solution (BioRad)
- 2-mercaptoethonol
- DTT (Sigma-Aldrich)
- IAA (Sigma-Aldrich)
- Agarose
- Poly-L-lysine (Sigma-Aldrich)
- Nitrocellulose membrane (Whitehead Scientific)
- AMPS (Sigma-Aldrich)
- TEMED (Sigma-Aldrich)
- 30% Bis/Acrylamide
- Running gel buffer pH 8.8
- Spacer gel buffer pH 6.8
- ColorBurst™ Electrophoresis Marker (Sigma)
- Ethanol (Kimix)
- 3% Acetic acid
- 0.1% Sodium metabisulphite in 10mM HCl (Merck)
- SCC x4
- Centrifuge tubes (Kimix)
- Specimen jars (Kimix)
- Freeze-dryer flasks (United Scientific)
- Polyclonal AGP (Abcam)

2D SDS-PAGE Electrophoresis:

- Coomassie Brilliant blue
- Acetone
- 8M urea buffer
- DTT
- IAA
- Ampholytes
- Bromophenol blue
- Mineral oil
- IPG strips (BioRad)
- Precast gels (Vacutec)
- PageRuler^{TM} Unstained Protein Ladder (Thermo Scientific)

Histochemistry and Immunohistochemistry:

- Formalin
- Xylol (Kimix)
- Absolute alcohol (Kimix)
- Haematoxylin (Merck)
- Scotts water
- 1% Eosin (Merck)
- Entellan (Merck)
- High iron diamine solution (Merck)
- 1% Alcian blue (Sigma-Aldrich)
- Acetone
- 1% Hydrogen peroxide
- Citrate buffer pH 6
- Normal goat serum (Dako)
- Polyclonal AGP (Abcam)
- Monoclonal MUC1 (Novocastra)
- Monoclonal MUC1core (Novocastra)
- Monoclonal MUC2 (Novocastra)
- Monoclonal MUC4 (Santa-Cruz)
- Monoclonal MUC5AC (Novocastra)
- Monoclonal MUC6 (Novocastra)
- Secondary antibody Envision monoclonal anti-mouse (Dako)
- Secondary antibody Envision polyclonal anti-rabbit (Dako)
- DAB (Dako)
- 1% Copper sulphate
- Coverslips (Lasec)

ELISA Assay:

- AGP Human ELISA kit (Abcam)
- MUC1 antibody (Abcam)
- MUC4 antibody (Abnova)
- MUC5AC antibody (Abcam)
- MUC6 antibody (Novus Biologicals)
- Polyclonal anti-mouse antibody (HRPO-linked) (Dako)
- Polyclonal anti-rabbit antibody (HRPO-linked) (Dako)
- PBS
- PBS with 0.5% Tween (PBST)
- 0.5% milk in PBS
- 1% TMB in DMSO (Merck)
- Citrate buffer pH 6
- 30% Hydrogen peroxide (Kimix)
- 2.5M Sulphuric acid
- 96-well plates (Lasec)

2.3 Methodology

2.4 Patients

Mucus scrapes (n=10) and blood samples (n=14) were obtained from patients who underwent a partial or total resection for malignant gastric disease. These patients were from Groote Schuur Hospital and the University of Cape Town Private Academic Hospital. The patient's information records are summarised in Table 2.1.

Lab assigned number	Age	Gender	Specimen	Type of Cancer	TNM Staging	Procedure	Hospital
2	55	M	Metastasis	Gastric adenocarcinoma	Stage IV	Stenting	GSH
3	65	M	No metastasis, 40mm x 30mm x 10mm in tumour size	Intestinal adenocarcinoma	Stage I	Partial gastrectomy	GSH
4	79	M	Metastasis	Gastric intestinal adenocarcinoma	Stage II	Full gastrectomy	GSH
5	72	F	Poorly differentiated carcinoma, multiple fragments.	Gastric adenocarcinoma	Stage IV	Unresectable	GSH
6	75	F	Metastasis	Gastric adenocarcinoma with gastric outlet obstruction	Stage IV	Unresectable, Stenting	GSH
7	25	F	Metastasis, diffuse carcinoma	Gastric adenocarcinoma	Stage IV	Stenting	GSH
8	61	M	Metastasis	Gastric adenocarcinoma with gastric outlet obstruction	Stage IV	Unresectable, Stenting	GSH
12	42	F	Metastasis, multiple fragments	Gastric adenocarcinoma with gastric outlet obstruction	Stage IV	Unresectable, Stenting.	GSH
15	79	M	No metastasis	Gastric adenocarcinoma	Stage I	Partial gastrectomy	GSH

Files from patients 1, 9-11 and 14 were not available (UCT Private and GSH).

Table 2.1: Clinical and pathological data of patients in this study.

2.5 Sample collection

2.51 Crude mucus scrapings

Total or partial gastrectomy samples (n=10) were provided shortly after resection by a surgeon. A small cut was made along the greater curvature of the stomach to expose the inner mucosal lining. Two sterile glass slides were used to gently scrape off the mucus from the mucosa into containers with 6M guanidinium chloride and a cocktail of protease inhibitors, namely, 5mM NEM, 10mM EDTA and 1mM PMSF pH 6.5. The samples were transported on ice and stored at -20°C until required.

2.52 Blood sample collection

Blood samples were taken from patients three days after surgery (n=14) by an intern. Normal blood was collected from volunteers by Dr Masheko Tetshedi, a qualified physician (n=15). The samples were collected into heparin tubes and spun at 3 000 rpm for 15 minutes at 4°C to separate the plasma from the blood cells. The plasma was then stored at -20°C until further examination.

2.53 Gastric tissue block collection

Tissue blocks that had been fixed with formalin and embedded with wax were obtained from the archives of the Division of Anatomical Pathology, National Health Laboratory Service, Groote Schuur Hospital. Professor D. Govender (pathologist) assisted in finding and collecting the available blocks (n=4) and in the assessment of the histochemistry and immunohistochemistry techniques.

2.6 Gastric scrape mucus purification

Crude mucus was stirred overnight at 4°C to solubilize the material. Samples were then homogenized, using a Junkel and Kunkel Ultra-Turrax by slowly increasing the revolutions from 8 000 rpm to 9 500 rpm at room temperature, to increase the solubility of the mucus for 30 seconds. The insoluble debris was removed by centrifugation at 3 000 rpm for 60 minutes at 4°C. The soluble mucus material was adjusted with 4M GuHCl and solid CsCl to a density of 1.39-1.40 g/ml (Creeth et al. 1970). Each sample was pipetted into a Beckman ultracentrifuge tube using a glass pasteur pipette and sealed using a Beckman tube topper. Isopycnic density gradient ultracentrifugation was performed for 48 hours at 40 000 rpm at 4°C (Beckman L45 centrifuge). The samples were divided into nine equal fractions and the density, protein and glycoprotein content of each fraction was determined. Each fraction was dialysed against distilled water three times over 24 hours at 4°C to remove CsCl and GuHCl.

The PAS positive glycoprotein fractions were pooled and subjected to a second isopycnic density ultracentrifugation and each fraction was assessed for density, protein and glycoprotein. The fractions with purified mucins (glycoprotein peak and density of 1.39-1.40 g/ml) for each sample were pooled, dialysed against 3 changes of distilled water and then freeze dried.

2.7 Analysis of fractions

2.71 Dialysis

The cellulose membrane dialysis tubing (Sigma-Aldrich Steinheim, Germany) was prepared by boiling in distilled water with 0.5% sodium bicarbonate/EDTA for 1 minute. Each fraction was then dialysed over 24 hours against three changes of distilled water. This was done to remove the GuHCl and the caesium chloride salts from the fractions.

2.72 Density determination

The density was determined by measuring the weight of 1.0 ml of each sample fraction against the weight of 1.0 ml of distilled water.

2.73 Protein content

The protein content was determined using the Bradford assay method (Bradford 1976) at an absorbance wavelength of 595nm using an Anthos htll Spectrophotometer, Austria.

2.74 Glycoprotein content

The periodic acid Schiff assay was used to measure the glycoprotein content (Mantle et al. 1978). Schiff reagent was decolourised by the addition of sodium metabisulphite and incubation in a water bath at 37°C for 1 hour. This was added to the sample after oxidation with periodic acid and then incubated for 30 minutes. The absorbance was read at 585nm.

2.75 Freeze drying

After dialysis, the samples were placed into containers with numerous smalls holes on the lids. Thereafter they were either frozen in a BIOVAC Ultra Low Freezer at -86°C or flash frozen in liquid nitrogen then transferred into Virtis vacuum flasks. These flasks were then connected to the freeze dryer (Christ Alpha I-5, Lasec) and the samples were left to freeze dry. The flasks were checked frequently to ensure the samples were not thawing as this would result in loss of sample.

2.8 1D SDS Polyacrylamide Gel Electrophoresis

The samples were reduced with 10mM DTT in 10mM PBS pH 6.5 and incubated in a 37°C water bath for 5 hours. The sulphate bonds were alkylated by the addition of 2.5mM of IAA per sample and left in the dark overnight.

The reduced and alkylated sample was diluted with sample application buffer containing 0.01% bromophenol blue, 2% sodium dodecyl sulphate, 10% glycerol and 0.2M 2-mercaptoethonol. The samples were boiled for 2 minutes, 15-20µg was loaded on a SDS-PAGE gel (7.5% running gel and 3.0% spacer gel) and run at 20mA per gel at 400V for approximately 2 hours on the Vacutec gel dock apparatus using the Laemmli method (Laemmli 1970). A molecular weight marker was included.

2.8.1 PAS Gel Staining

After electrophoresis, the gel was stained with PAS according to the method of Dubray and Bezard. Gel fixation in 50% ethanol then oxidation in 1% periodic acid in 3% acetic acid was performed. Thereafter the gel was stained with Schiff reagent in the dark and destained with 0.1% sodium metabisulphite (Dubray et al. 1982).

2.9 2D Proteomics

2.9.1 Sample protein precipitation and preparation

The gastric mucus scrapes that had a positive pink spot on the 1D SDS-PAGE where chosen for 2D electrophoresis. Five mg of each sample was dissolved in 2.0 ml of PBS and the proteins were precipitated using four volumes (8.0 ml) of acetone. The samples were left overnight at -20°C then spun at 3 000 rpm at 4°C for 15 minutes. The supernatant was removed and the pellet resuspended in 8M urea buffer at room temperature. A Bradford assay was performed with protocol described earlier in section 2.73. The volume equivalent to 100µg was calculated for each sample.

2.9.2 Reswelling/Rehydrating the IPG strips

To each sample 50% DTT then 1.25µl ampholytes was added and made up to a volume of 125µl with 8M urea buffer. Bromophenol blue was added to the sample and centrifuged. The reswelling tray was balanced on a level surface then each sample was added into a separate tray lane. The immobiline pH gradient (IPG) strips (7cm, pH 3-10, BIO-RAD) were removed from the fridge and the gel was carefully separated from the cover slip. Each gel was then placed on the sample with the gel side facing downwards. Care was taken not to trap bubbles under the gel strip. Drystrip Cover Fluid (GE Healthcare) mineral oil was poured into each lane to ensure the gel strip did not dry out. The samples were left to reswell overnight at room temperature.

2.9.3 Isoelectric focusing of the IPG strips

After rehydration, the strips were rinsed in distilled water to remove excess urea buffer which can cause bad resolution on the gels as well as increase the time of isoelectric focussing (IEF) if urea recrystallisation occurs. The strips were then placed on the IEF machine (Ettan^{TM}IPGphorrII^{TM} IEF machine, GE Healthcare, Amersham, UK), gel side facing upwards with 1.5cm wicks at either end of each strip covering a small portion of the gel. The electrodes were placed on the wick and thereafter each lane was covered with mineral oil. The machine was then run in a step wise programme with the following settings for approximately 6 hours at 20°C: Step 1 – 250V for 15 minutes; Step 2 – 4 000V for 1 hour; Step 3 – up to 12 000Vh.

2.10 2D SDS-PAGE

After IEF, the strips were washed with distilled water and blotted on wet filter paper. The strips were equilibrated by incubating them in an SDS buffer (6M urea, 2% SDS, 50mM Tris/HCl pH 8.8 and 20% glycerol) containing 2% DTT for 15 minutes on a shaker. Following this, the strips were treated with SDS buffer containing 2.5% iodoacetamide in place of DTT. The strips were then washed with 1 x SDS-PAGE running buffer to aid in the transfer of proteins from the IPG strips to the SDS-PAGE. The strips were then loaded onto a pre-cast 12% SDS-PAGE. A marker was added to each gel on a dried 0.5cm wick then the strips were sealed with 0.5% agarose which solidified before beginning the electrophoresis. The gels were run at 130V for approximately 75 minutes at room temperature until the dye had reached the bottom of the plates. The gels were stained with PAS (described previously) and Coomasssie Brilliant Blue to visualise the protein spots.

2.10.1 Coomassie Brilliant Blue R-250 gel staining

After gel electrophoresis, the gels were stained with Coomassie solution 1 (10% glacial acetic acid, 2% Coomassie stock, 25% propan-2-ol) for 30 minutes after heating in the microwave for approximately 1 minute. This was repeated with Coomassie solution 2 (10% glacial acetic acid, 0.25% Coomassie stock, 10% propan-2-ol) and Coomassie solution 3 (10% glacial acetic acid and 0.25% Coomassie stock). Thereafter the gels were destained in destaining solution (10% glacial acetic acid, 1% glycerol in water) until the protein spots become visible.

2.10.2 Protein spot identification

The target protein spots were identified according to size using the molecular weight marker and the corresponding PAS staining as reference. Each spot was excised with a clean tip and sent for identification using MALDI-TOF-TOF mass spectrometry. Briefly: The spot was excised then underwent reduction with 50mM DTT then alkylation with 50mM IAA. The reduced and alkylated spot was then digested with Trypsin Gold (Promega, Madison USA) at 37°C for 15 hours. Then 8µl 10% trifluoroacetic acid (TFA) was added to the digested spot and transferred to 4°C to stop the digestion and stored at -20°C until needed. The digests were extracted from the gel and analysed on the UltrafleXtreme MALDI-TOF-TOF mass spectrometer (Bruker, Bremen) using a matrix (alpha-cyano-4-hydroxy-cinnamic acid, CHCA) mixed on a prespotted PAC-II 386 spot target plate (Bruker). The AutofleX script was executed to run MS and tandem MS in reflectron mode where the top 50 precursor

peptide ions were selected from the spectra. Laser-induced fragmentation was then used for further sequencing to generate MS/MS spectra. For peptide identification, the MS/MS spectra corresponding to each sequenced precursor ion of given m/z value was searched for on the Mascot search engine in the Bruker ProteinScrape server. This was searched against in-silico MS/MS spectra generated using the Swissprot 2013_01 database. Spectra to peptide matches were produced on the basis of the search parameters for our protein of interest which identified the spot of interest.

2.11 Enzyme-linked Immunosorbent Assay (ELISA)

For the quantitative measurement of human AGP concentrations in plasma from normal healthy individuals and cancer patients (done in duplicate), an alpha-1-acid glycoprotein Human ELISA kit (abcam) was used. The AGP concentrations were determined using the protocol from the kit and read from the standard curve generated by using the standard provided. Statistical analysis was performed using the StatPlus package from Analystsoft. Standard deviations were calculated as for a sample population.

For semi-quantitative measurement of the mucins, a sandwich ELISA was used with plasma samples from healthy individuals and cancer patients. The target antigens were MUC1, MUC4, MUC5AC and MUC6 and the respective antibodies were used. The ELISA was optimised with the following parameters: pH of the carbonate coating buffer, concentration of the primary antibody and the concentration of the antigen. After optimisation, the primary antibody for each antigen was diluted at the optimal concentration (shown in Table 2.2) and incubated overnight at 4°C in a 96-well plate and the contents discarded. Next, blocking buffer (0.5% gelatine in PBS) was added to each well to minimise non-specific binding, the plate was sealed and incubated at room temperature for 4 hours. The well contents were discarded and the plasma from each patient was then added to the 96-well plate in triplicate. The plasma samples for MUC1, MUC4, MUC5AC and MUC6 were diluted in PBST with 0.1% gelatine at concentrations of 1:200, 1:500, 1:200 and 1: 200 respectively. The plate was then incubated overnight at 4°C. The unbound sample was discarded and thereafter each well was washed with washing buffer (PBST with 0.1% gelatine) three times. Detection was performed with a secondary antibody (HRP antibody) which bound to the antigen. The secondary antibody was diluted according to the manufacturer's protocol and added to each well. After 2 hours incubation at room temperature, the well contents were discarded, washed again with PBST and working substrate solution (1% TMB in DMSO, citrate buffer pH 6.0 and 30% H_2O_2) added to each well for 20 minutes at 37°C. The colour reaction was stopped by the addition of 2.5M H_2S0_4 and the OD of the samples read at a wavelength of 405nm.

Antibody	Antigen	Concentration
Mouse monoclonal MUC1 (Abcam)	Peptide epitope RPAP within protein core	1:100
Mouse monoclonal MUC4 (Abnova)	Partial recombinant protein of MUC4	10µg/100µl
Mouse monoclonal MUC5AC (Abcam)	Human MUC5AC	1:100
Rabbit polyclonal MUC6 (Novus Biologicals)	C terminus region of protein MUC6	1:100

Table 2.2: Mucin antibodies used in ELISA.

2.12 Histological studies

2.12.1 Sample preparation

Gastric tissue blocks from patients who had a gastrectomy were retrieved from the archives of the Division of Anatomical Pathology, National Health Laboratory Service, Groote Schuur Hospital. The gastric tissue was formalin fixed and paraffin wax embedded. Two µm sections were cut using a microtome (Leica SM 2000 R, Germany). The sections were mounted onto glass slides for histochemistry and amino-propyl-tri-ethoxy-silane (APES) slides for immunohistochemistry. Positive and negative tissue controls were cut for each antigen as well as normal gastric tissue. The slides were incubated at 55°C for 40 minutes then left at room temperature until staining.

2.12.2 Histochemistry

The section from each patient was stained with haematoxylin and eosin (H&E) which shows morphology, determining diagnosis. Sections were stained with periodic acid Schiff/alcian blue (pH 2.5) (PAS/AB) to identify acidic (blue) and neutral (pink) mucins. Sections were also stained with high iron diamine/alcian blue (HID/AB) which stains sulfomucins (black) and sialomucins (blue). The protocols described in Bancroft and Gamble 2008 were followed for the H&E and PAS/AB stain and Spicer 1965 for HID/AB staining.

2.12.3 Immunohistochemistry

A general protocol was followed where each tissue sample available was tested once for each antigen but different incubation times were used for the primary antibody depending on the antigen. The sections were dewaxed in three changes of xylol for 5 minutes each, and then rehydrated through decreasing graded alcohols for 2 minutes each. After rinsing in water, the slides were incubated at room temperature in 1% H_2O_2 for 15 minutes to block endogenous peroxidise activity. Following this, heat-mediated antigen retrieval was performed in citrate buffer (0.01M, pH 6.0) in a pressure cooker (Amalgamated Appliances Holdings, Reuven, South Africa) for 2 minutes at full pressure. The slides were then cooled in running tap water for 10 minutes then rinsed with PBST. Next blocking was performed using normal goat serum in PBS for 10 minutes (1:20) to prevent non-specific binding. A negative control was added replacing the primary antibody with PBS. The primary antibody diluted in PBS was added to the slides. The concentration of the primary antibodies and the varying incubation times as well as the relevant positive control tissue is shown in Table 2.3. After incubation, each slide was washed with PBST then incubated with secondary antibody (Envision), either anti-mouse or anti-rabbit depending on the primary antibody, for 30

minutes. After rinsing the colour was developed with DAB for 8 minutes. DAB was prepared by mixing 1 drop of DAB per 1.0 ml of substrate buffer. The reaction was stopped by rinsing the slides in PBST then distilled water and thereafter the colour was enhanced with 1% copper sulphate for 10 minutes. Haematoxylin was used to counterstain for 2 minutes then in Scotts water for 2 minutes for blueing. After rinsing in running tap water for 5 minutes the sections were dehydrated in increasing graded alcohols for 10 seconds each, then cleared in xylol. The sections were cover slipped using Entellan and left to dry before viewing under a light microscope.

2.12.4 Immunostaining scoring

A semi-quantitative method was used to evaluate tissue expression of AGP and the mucins. Each section was examined by Professor D. Govender at x40 objective and scored as follows: <5% non stained cells = 0; 5-25% stained cells = 1+; 26-50% stained cells = 2+; 51-75% stained cells = 3+; >75% stained cells = 4+.

Antibody	Antigen	Dilutions, incubation time	Positive tissue controls
Rabbit polyclonal anti-alpha-1-acid glycoprotein (Abcam)	Human Alpha-1-acid glycoprotein	1: 1000 (30 min)	Normal gastric
Mouse monoclonal anti-MUC1(Nova castra)	Peptide epitope RPAP	1:100 (30 min)	Breast carcinoma
Mouse monoclonal anti-MUC1core (Novo castra)	TRTPAPG sequence in tandem repeat region in glycoprotein	1:100 (30 min)	Breast carcinoma
Mouse monoclonal anti-MUC2 (Nova castra)	Human MUC2 mucin	1:100 (1 hr)	Normal colon
Mouse monoclonal anti-MUC4 (Santa Cruz Biotechology Inc)	Peptide epitope STGDTTPLPVTDTSSV in tandem repeat region	1:100 (overnight at 4°C)	Normal colon
Mouse monoclonal anti-MUC5AC (Novo castra)	MUC5AC tandem repeat sequence	1:100 (1 hr)	Normal gastric
Mouse monoclonal anti-MUC6 (Novo castra)	Human MUC6 glycoprotein	1:100 (1 hr)	Normal gastric
2° antibody, goat anti-rabbit, HRPO-linked antibody		Envision (30 min)	
2° antibody, goat anti-mouse, HRPO linked antibody		Envision (30 min)	

Table 2.3: Antibodies and control tissues used in immunohistochemistry.

Results and Discussion

This project aimed to investigate whether alpha-1-acid glycoprotein is a suitable clinical biomarker candidate for pre-malignant gastric cancer. Previous results found in our lab showed that a PAS positive 55-65kDa glycoprotein associated with albumin was reproducibly detected on SDS-PAGE of purified mucins in gastric disease, namely carcinoma of the stomach (Mall et al. 1990; Mall et al. 1992). The glycoprotein was also found in the gastric juice of patients diagnosed with cancer of the stomach (Mall et al. 2000) and the albumin was separable from the glycoprotein on column chromatography. Subsequent studies found that this glycoprotein was actually alpha-1-acid glycoprotein (Chirwa et al. 2012) and not a fragment of MUC5AC as was previously reported (Mall et al. 1999). The identification of the glycoprotein by Chirwa et al (Chirwa et al. 2012) was restricted to a MALDI-TOF analysis of 3 spots on a 2D-gels stained with Coommassie Blue (Chirwa et al. 2012). The PAS stained spot had not been identified with the possibility of it being another glycoprotein other than α-1-acid glycoprotein.

In this project, we sought firstly to confirm the identity of this glycoprotein. The concentration of AGP in the blood plasma of cancer patients was then determined and compared with that from healthy controls using ELISA. We correlated the concentrations of AGP to the protein levels of MUC1, MUC4, MUC5AC and MUC6. This was done to determine whether there was any association between blood AGP levels with gastric mucins. This was a study to determine the possibility of there being a combination of markers, namely AGP and one or more of the gastric mucins. Immunohistochemistry was also carried out to determine protein expression levels of AGP, MUC1, MUC1c, MUC2, MUC4, MUC5AC and MUC6 and to correlate these results with the ELISA findings.

Chapter three – Mucus purification and glycoprotein identification

3.1 Mucus purification

Crude gastric mucus was collected in 6M GuHCl containing protease inhibitors to prevent endogenous proteolysis. The crude mucus was purified twice by isopycnic density gradient ultracentrifugation with caesium chloride and 4M GuHCl at a density of 1.39 – 1.40 g/ml to remove nucleic acids and protein contaminants. The protein content was determined by the Bradford method (Bradford 1976) and the mucin content by the PAS method of Mantle and Allen (1978) (Figure 3.1). The PAS positive peak was collected (density 1.39-1.40g/ml) and subjected to another density gradient spin, after which purified mucins were obtained (Creeth et al. 1970; Habte et al. 2006). The pool of purified mucins were dialysed overnight against 3 changes of distilled water and freeze-dried. The purification process is important to allow for clear separation during gel electrophoresis.

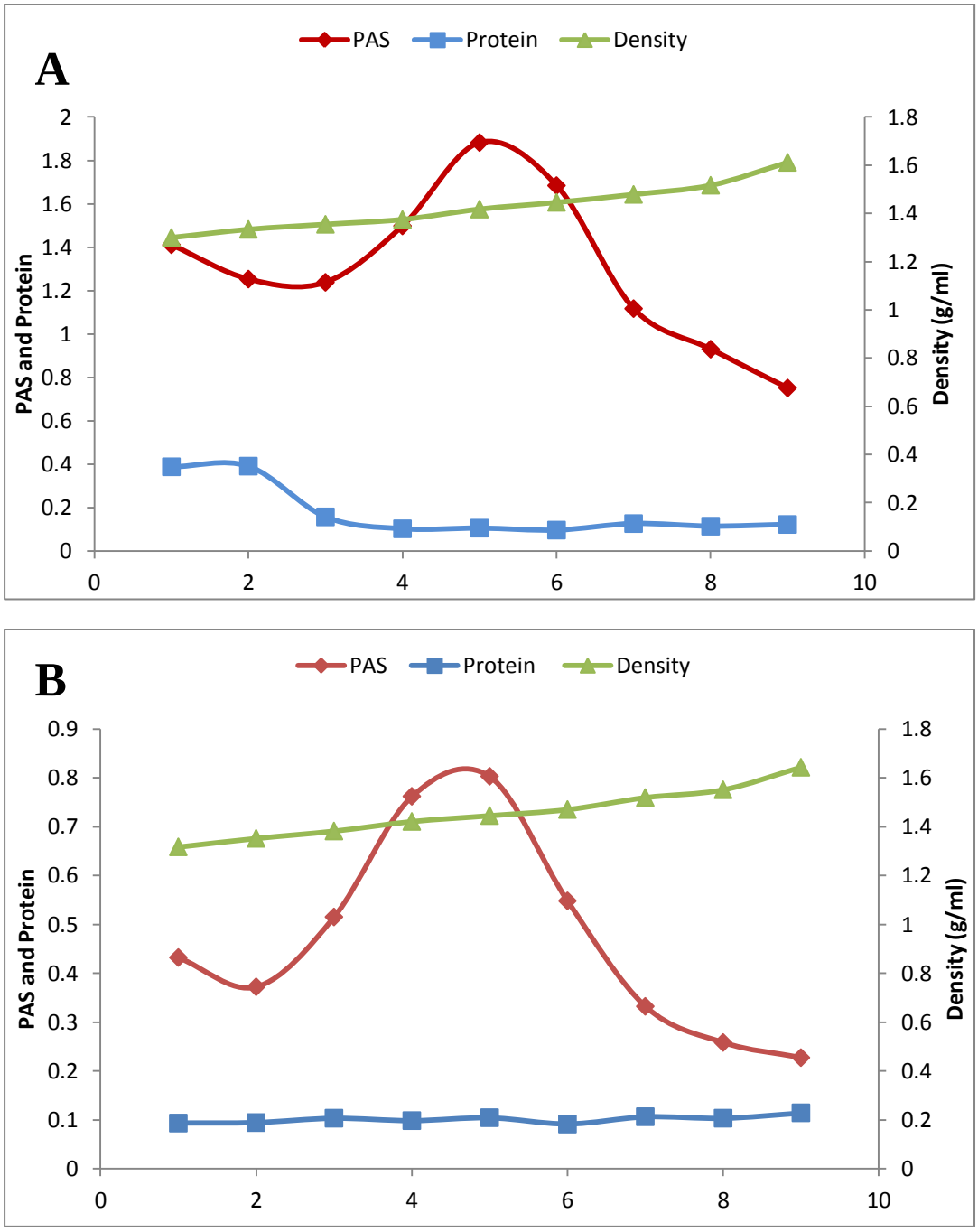


Figure 3.1: Isopycnic ultracentrifugation profiles of gastric mucus purification.

Crude gastric mucus was dissolved in 6M GuHCl with 10mM EDTA, 5mM NEM and 1mM PMSF pH 6.5 and the density was adjusted to 1.39 – 1.40 g/ml with caesium chloride. Mucins were purified by density gradient ultracentrifugation for 48 hours at 40 000 rpm. **A:** Mucins were PAS positive (◆) with a density (▲) between 1.39-1.40 g/ml and some protein (■) association. **B:** A second density gradient ultracentrifugation was performed and uncontaminated fractions (fraction 3-6) were pooled, dialysed against three changes of H_2O at 4°C overnight then freeze-dried.

3.2 Protein separation and glycoprotein staining

3.2.1 1D SDS-PAGE

The freeze dried samples of the purified mucins were reduced overnight with 10mM DTT and blocked with 2.5mM IAA. The proteins were then separated by molecular weight on a 10% 1D SDS-PAGE and stained with PAS which identifies glycoproteins by staining them pink. Figure 3.2 shows 1D SDS gels from patients 1-9. Five of these samples (shown by the circles) stained positively for glycoprotein content at approximately 40-50kDa. This correlates with previous results found in our laboratory (Chirwa et al. 2012) . Samples 8, 9 and 10 stained darkly for glycoprotein content indicating high levels of AGP compared to the other positive samples.

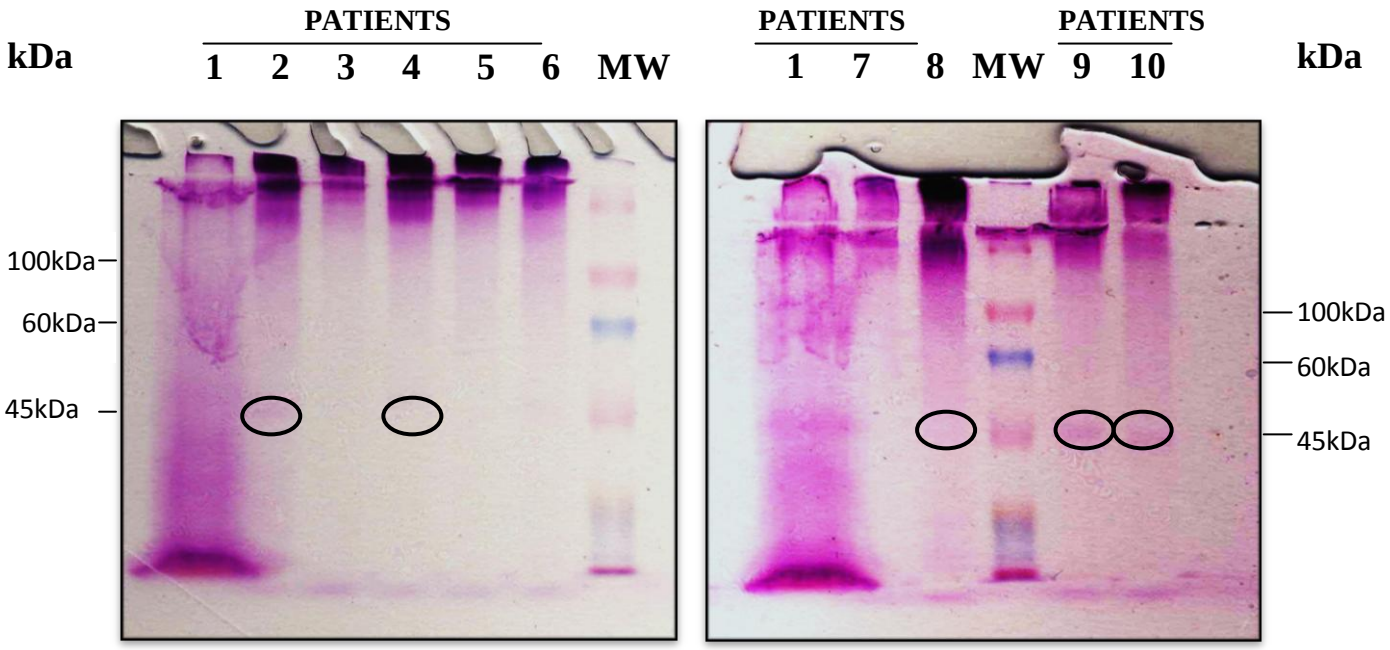


Figure 3.2: 1D SDS-PAGE of purified gastric mucins stained with PAS for glycoprotein identification.

Freeze dried purified mucin samples were reduced with 10mM DTT and blocked with 2.5mM IAA overnight then run on a 10% 1D SDS-PAGE at 400V per gel at 20mA for 2 hours with a molecular weight marker. Thereafter the gels were stained with PAS to detect glycoproteins. Patients 2, 4, 8, 9 and 10 (shown by ⬭) stained positive for AGP and could be examined on a 2D electrophoresis gel.

3.2.2 2D SDS-PAGE

3.2.2.1 PAS staining of the 2D SDS-PAGE

Purified mucus samples from patients 2, 4, 8, 9 and 10 stained positively for a glycoprotein at size 40-55kDa. Patient samples 4, 9 and 10 were used for glycoprotein identification as there was sufficient freeze dried material to be used for further experimentation. These samples were only separated according to molecular weight on the 1D SDS-PAGE thus enabling the identification of our protein of interest, they had to be further separated by the isoelectric point (pI) using 2D SDS-PAGE. Each sample was precipitated with acetone then resuspended in 8M urea and rehydrated on an IPG strip (immobiline pH gradient) (pH 3-10) overnight. After undergoing isoelectric focusing in a three stepwise program each sample was run on a 2D SDS-PAGE and stained with PAS to detect the glycoprotein spot equivalent to the 40-55kDa glycoprotein of interest. A 12% 2D SDS-PAGE is used to separate proteins according to their pI by isoelectric focussing (first dimension) then by the molecular weight by an SDS-PAGE (second dimension). This enables visualisation of proteins that differ by a single charge and can be excised precisely for identification. Figure 3.3 shows sample 4 and 9 (approximate pI between pH 3 and pH 4) and we observed a pink spot positively identifying glycoprotein at approximately 40-55kDa. Sample 10 showed very faint staining which did not photograph well. This may be attributed to low amounts of glycoprotein present in the sample. The molecular weight marker faded during PAS staining thus a picture of the marker taken pre-staining was included in the figure.

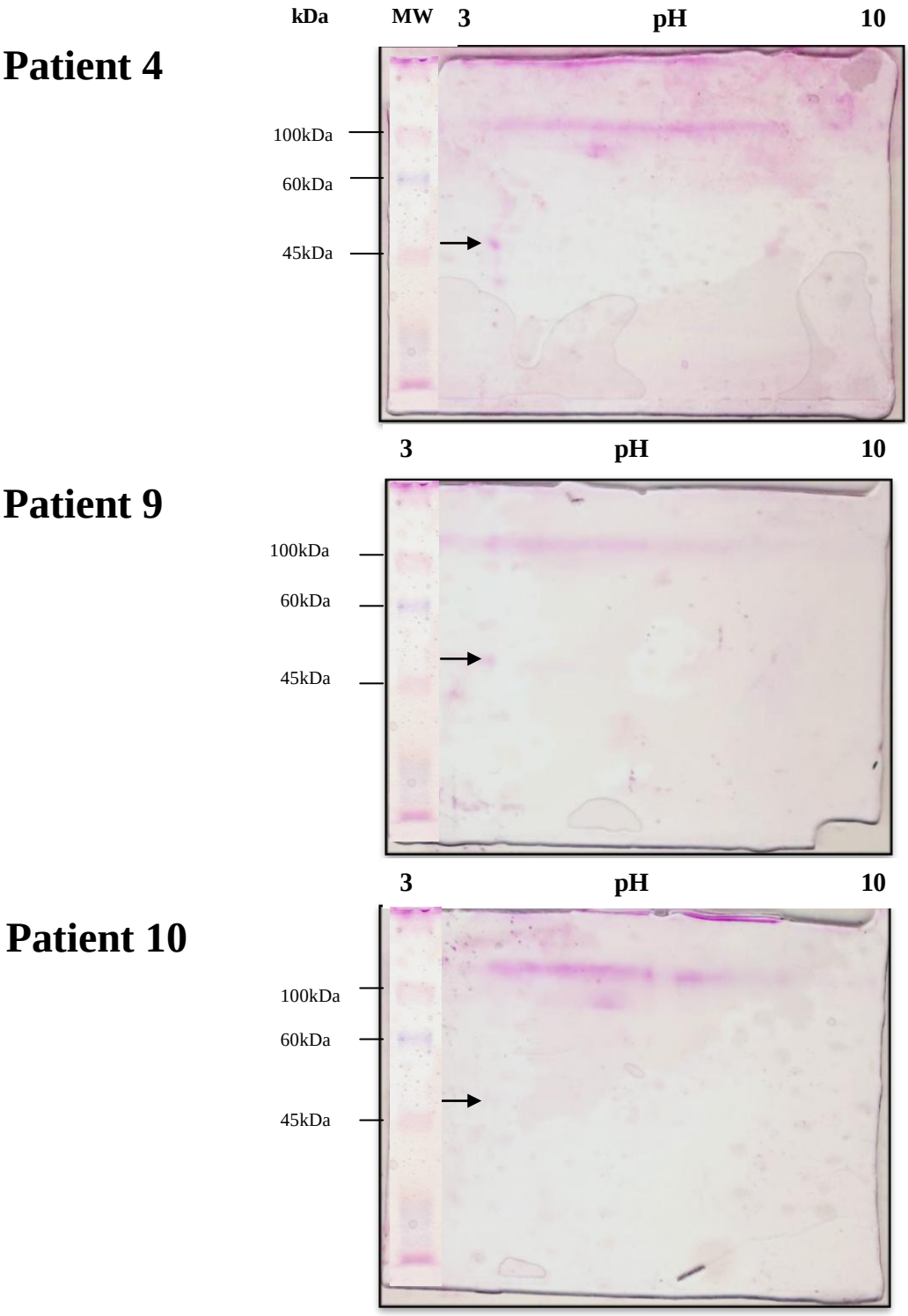


Figure 3.3: 2D SDS-PAGE of purified gastric mucins stained with PAS for glycoprotein identification.

Patients 4, 9 and 10 that stained positive for glycoprotein content on a 1D SDS-PAGE were run on 2D SDS-PAGE gels. The proteins from each sample were precipitated with acetone then resuspended in 8M urea buffer and one hundred micrograms of each sample was rehydrated on IPG strips overnight. The strips underwent isoelectric focusing at 12 000Vh and thereafter were run on a 12% SDS-PAGE at 130V for 75 min. The gels were stained with PAS to detect the presence of glycoproteins.

3.2.2.2 Coomassie Blue staining of the 2D SDS-PAGE

The process of separating the proteins two-dimensionally was repeated with samples 4, 9 and 10 but this time the gels were stained using Coomassie Brilliant Blue. This was done to enable the identification of the protein spot of interest for MALDI-TOF-TOF analysis as the Coomassie Blue stain is compatible with this process. Figure 3.4a shows the gel stained from sample 4 with Coomassie Blue before the spots of interest (shown by the arrow) were excised from the gel. A large amount of protein spots are observed on the gel including our protein of interest which was highly expressed indicated by the dark staining. Our protein has a size of ~40-50kDa on this picture which corresponds to the band observed in the 1D SDS-PAGE shown in Figure 3.2.

Figure 3.4b shows the Coomassie Blue stained gel from sample 9 which was also run on a 2D SDS-PAGE. This gel differs to the gel shown in Figure 3.4a as there is a reduction in the number of overall protein spots observed. The protein of interest is shown with an arrow at the size ~40-50kDa with high staining intensity indicating high expression, similar to sample 4.

Sample 10, Figure 3.4c again shows a similar Coomassie Blue stained gel to Figure 3.4a and 3.4b except it has even less protein spots visible. The spot of interest is shown with the arrow and it can be observed that though it is the size of our glycoprotein at ~40-50kDa, it stained very faintly indicating low expression.

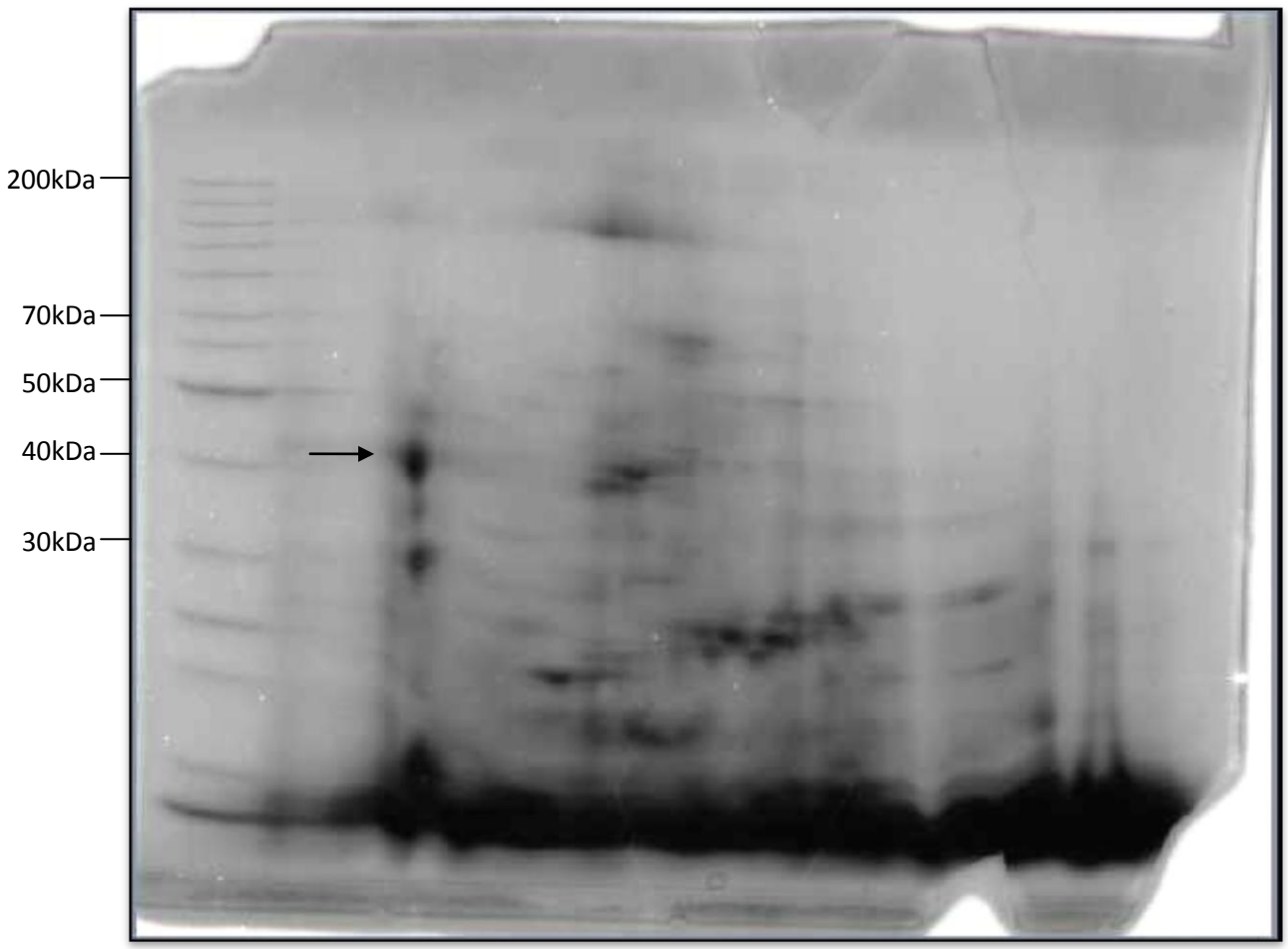


Figure 3.4a: 2D SDS-PAGE of purified gastric mucins stained with Coomassie Blue for protein identification in sample 4.

Sample 4 stained positive for glycoprotein content on a 1D SDS-PAGE and was run on a 12 % 2D SDS-PAGE gel. The proteins in the sample were precipitated with acetone then resuspended in 8M urea buffer. One hundred micrograms of the sample was rehydrated on an IPG strip overnight. The strip then underwent isoelectric focusing up to 12 000Vh and thereafter run on a SDS-PAGE at 130V for 75 min. The gel was stained with Coomassie Brilliant Blue to visualise the protein spot of interest shown in Figure (⟶).

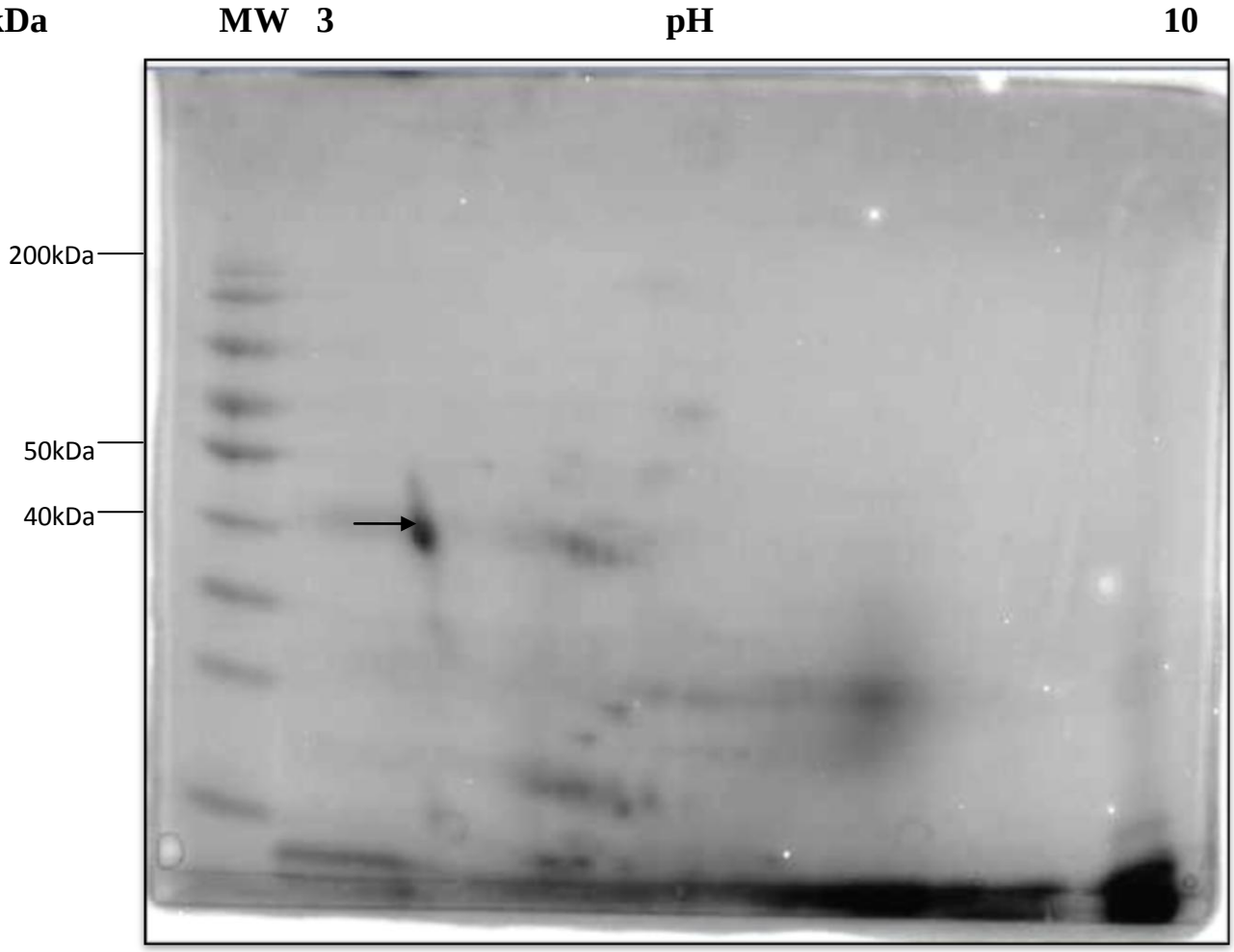


Figure 3.4b: 2D SDS-PAGE of purified gastric mucins stained with Coomassie Blue for protein identification in sample 9.

Sample 9 stained positive for glycoprotein content on a 1D SDS-PAGE and was run on a 12% 2D SDS-PAGE. The proteins in the sample were precipitated with acetone then resuspended in 8M urea buffer. One hundred micrograms of the sample was rehydrated on an IPG strip overnight. The strip then underwent isoelectric focusing up to 12 000Vh and thereafter run on a SDS-PAGE at 130V for 75 min. The gel was stained with Coomassie Brilliant Blue to visualise the protein spot of interest shown in Figure (⟶).

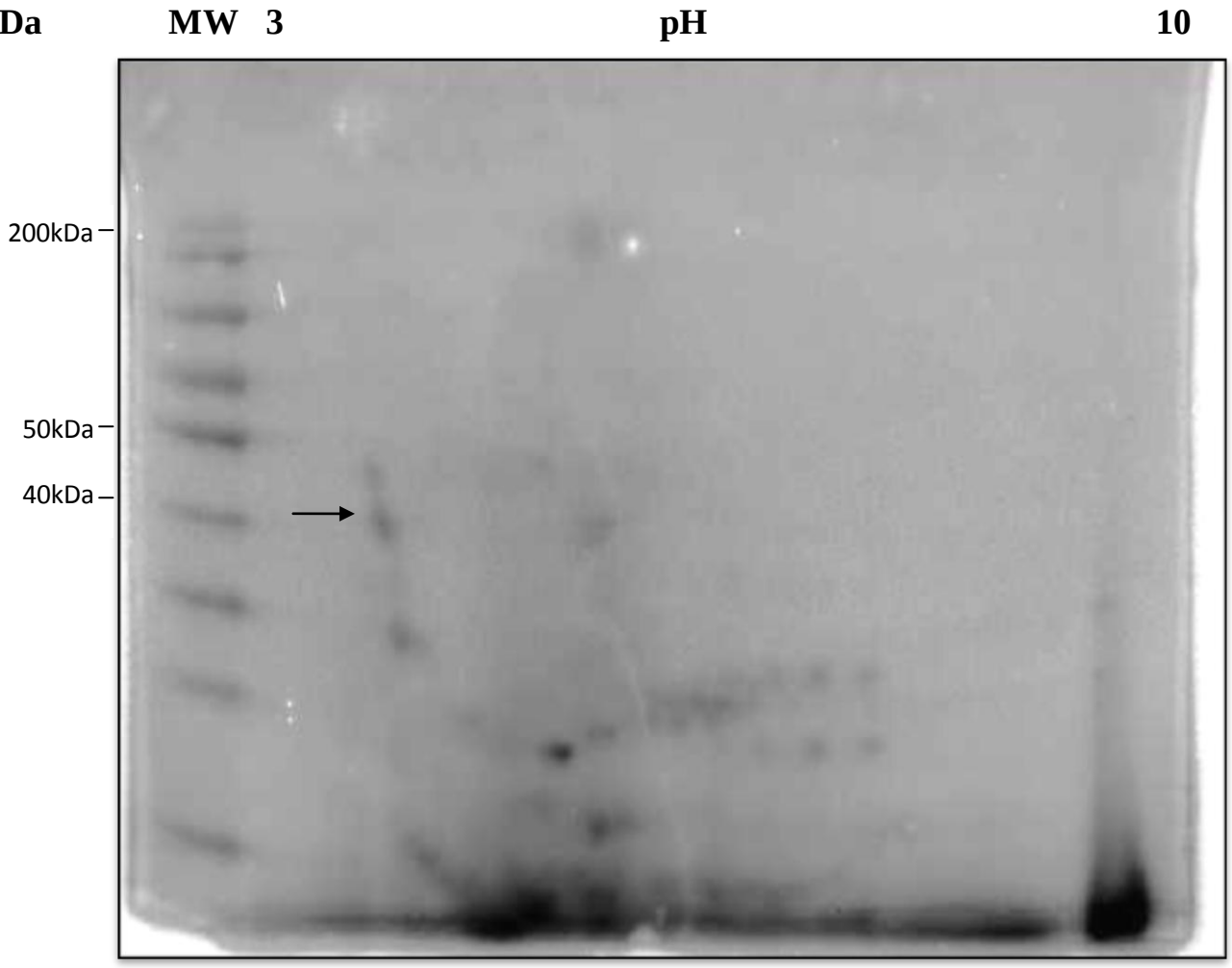


Figure 3.4c: 2D SDS-PAGE of purified gastric mucins stained with Coomassie Blue for protein identification in sample 10.

Sample 10 stained positive for glycoprotein content on a 1D SDS-PAGE and was run on a 12% 2D SDS-PAGE gel. The proteins in the sample were precipitated with acetone then resuspended in 8M urea buffer. One hundred micrograms of the sample was rehydrated on an IPG strip overnight. The strip then underwent isoelectric focusing up to 12 000VHrs and thereafter run on a SDS-PAGE at 130V for 75 min. The gel was stained with Coomassie Brilliant Blue to visualise the protein spot of interest shown in Figure (⟶).

3.3 Glycoprotein Identification

3.3.1 Spot Excision

After the proteins were separated two dimensionally and stained with Coomasie Brilliant Blue, the spot of interest (shown with an arrow, Figure 3.4a-c) was excised. The spot was then reduced with DTT, alkylated with IAA and digested with trypsin to produce peptides. The peptide digest was analysed on the UltrafleXtreme MALDI-TOF-TOF mass spectrometer (Bruker, Bremen). The ions generated were analysed by their mass/charge ratio and a list of peptide and amino acid masses (peak lists) were used to search and identify proteins in Matrix Science Algorithms.

3.3.2 Mass Spectrometry Analysis

Proteins were identified by the probability-based molecular weight search (MOWSE) score after they were searched through the SwissProt 2013_01 protein database using the Mascot search engine. The protein MOWSE score was calculated as $-10^{x}\text{Log}(P)$, where P is the absolute probability of matching a number of peaks by random chance with a significance level of $p<0.05$. Table 3.1 shows a summary of the identified proteins from sample 4, 9 and 10 using the above parameters.

The Mascot search results yielded a histogram for each sample whereby the height of each bar reflects the number of protein matches. Sample 4 had an overall MOWSE score of 49 ($p<0.05$) and six protein hits identified the protein as alpha-1-acid glycoprotein, the Homo sapiens species (shown in Figure 3.5a). Figure 3.5b shows the overall protein sequence coverage which was 11%. This reflects the actual measured peptide coverage on the higest scoring polypeptide, an α-1-acid glycoprotein (A1AG1_Human PO2736) in the case of sample 4 and 9.

Figure 3.6a shows the Mascot search results run under the same parameters as with sample 4. This sample also had six protein hits but had an overall score of 71 ($p<0.05$) which reflects a more confident match than that of sample 4. Figure 3.6b shows that the protein sequence coverage was again 11% for the identified protein alpha-1-acid glycoprotein, Homo sapiens.

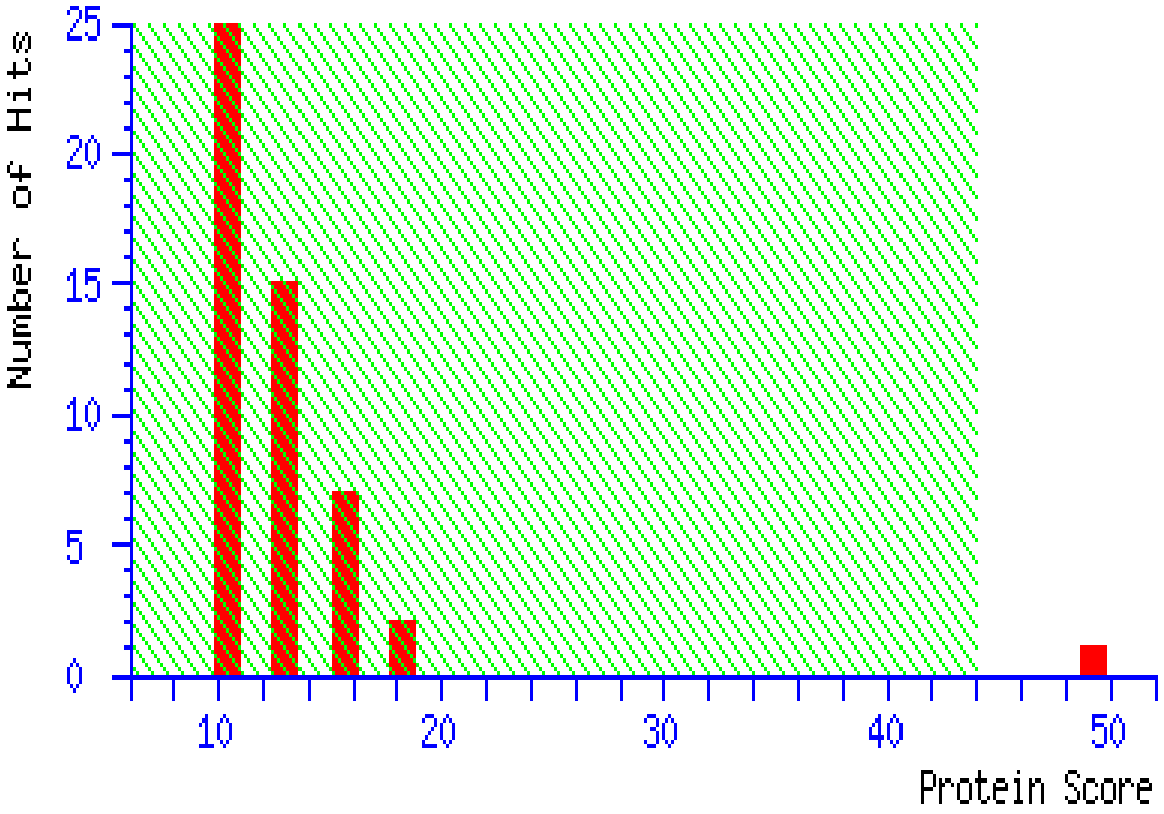
Sample 10 was identified as keratin, type II, cytoskeletal 1 shown in Figure 3.7 with three protein hits. Obviously this was a false result and keratin contamination may have occurred while running the SDS-PAGE and handling the gels, pipette tips and IPG strips thus we disregarded this result.

Each of the positive results had a *p* value less than 0.05 which made them significant therefore we confirmed previous results in our lab (Chirwa et al. 2012) that the pink spot observed when MUC5AC was fragmented (Mall et al. 1999; Mall et al. 1999) was indeed alpha-1-acid glycoprotein.

Sample number	Protein identified	Accession number	Theoretical monoisotopic Mass	Theoretical pI	MOWSE Score	Peptide Matches
Sample 4	Alpha-1-acid glycoprotein human	A1AG1_Human P02763	23725	3-4	49	6
Sample 9	Alpha-1-acid glycoprotein human	A1AG1_Human P02763	23725	3-4	71	6
Sample 10	Keratin-1, type-II human	K2C1_Human P04264	66170	3-4	65	3

Table 3.1: Summary of identified proteins from the SwissProt 2013_01 database using the Mascot search engine.

A

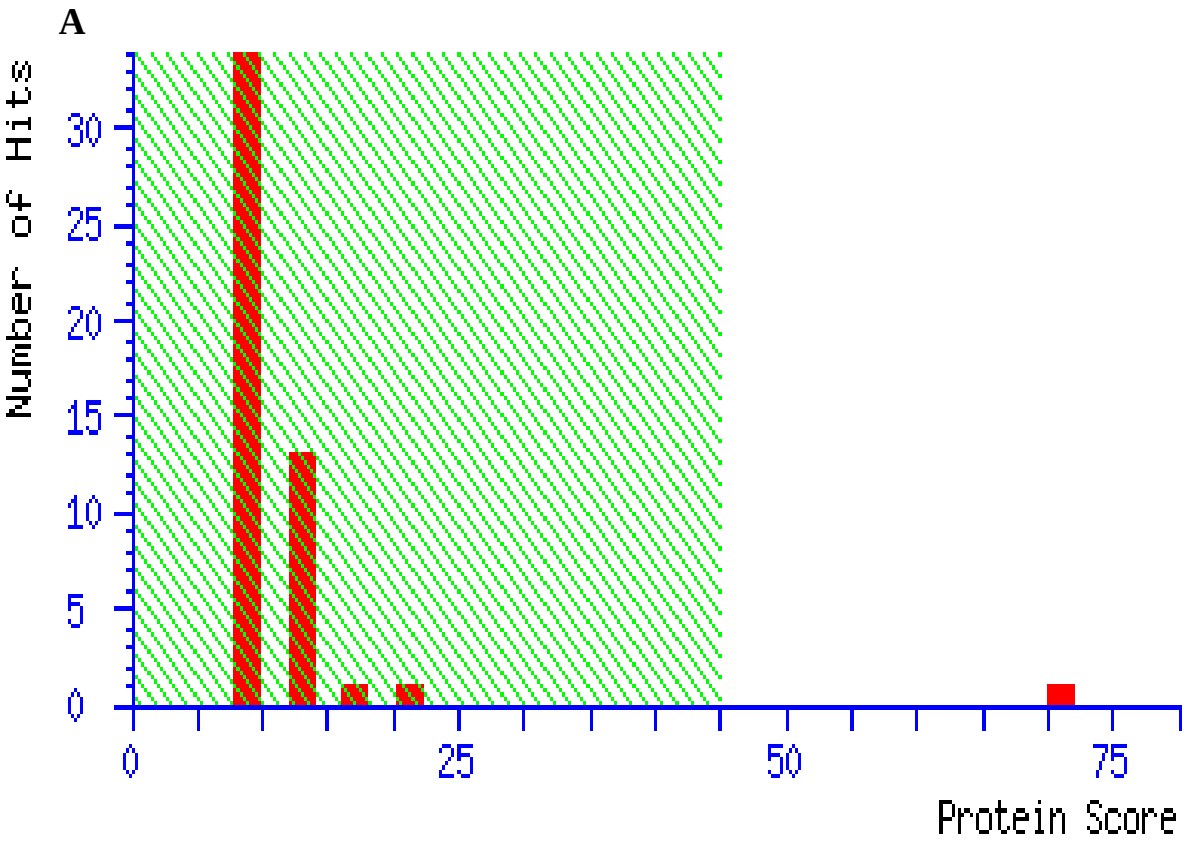


B

1	MALSWVLTVL	SLLPLLEAQI	PLCANLVPVP	ITNATLDQIT	GK**WFYIASAF**
51	**R**NEEYNKSVQ	EIQATFFYFT	PNKTEDTIFL	REYQTRQDQC	IYNTTYLNVQ
101	RENGTISR**YV**	**GGQEHFAHLL**	**ILR**DTKTYML	AFDVNDEKNW	GLSVYADKPE
151	TTKEQLGEFY	EALDCLRIPK	SDVVYTDWKK	DKCEPLEKQH	EKERKQEEGE
201	S				

Figure 3.5: Mascot, a histogram of the probability based score results and protein sequence coverage for protein identification from sample 4.

A: Mascot search results for protein spot (from sample 4) that was excised then run via MALDI TOF analysis for identification. The ion score is -10^{x}Log (P) where P is the probability that the match observed is a random event. Scores > 44 indicates extensive homology ($p<0.05$). The protein hits identified it as A1AG1, Homosapiens; Mass: 23725, Score: 49. **B:** The matched peptides from our protein spot are shown in **bold red** against the protein sequence of AGP. A protein sequence coverage of 11% was observed in sample 4 where $p<0.05$.



B

1	MALSWVLTVL	SLLPLLEAQI	PLCANLVPVP	ITNATLDQIT	GK**WFYIASAF**
51	**R**NEEYNKSVQ	EIQATFFYFT	PNKTEDTIFL	REYQTRQDQC	IYNTTYLNVQ
101	RENGTISR**YV**	**GGQEHFAHLL**	**ILR**DTKTYML	AFDVNDEKNW	GLSVYADKPE
151	TTKEQLGEFY	EALDCLRIPK	SDVVYTDWKK	DKCEPLEKQH	EKERKQEEGE
201	S				

Figure 3.6: Mascot, a histogram of the probability based score results and protein sequence coverage for protein identification from sample 9.

A: Mascot search results for protein spot (from sample 9) that was excised then run via MALDI TOF analysis for identification. The ion score is $-10^{x}\text{Log}(P)$ where P is the probability that the match observed is a random event. Scores > 44 indicates extensive homology ($p<0.05$). The protein hits identified it as A1AG1, Homosapiens; Mass: 23725, Score: 71. **B:** The matched peptides from our protein spot are shown in **bold red** against the protein sequence of AGP. A protein sequence coverage of 11% was observed in sample 4 where $p<0.05$.

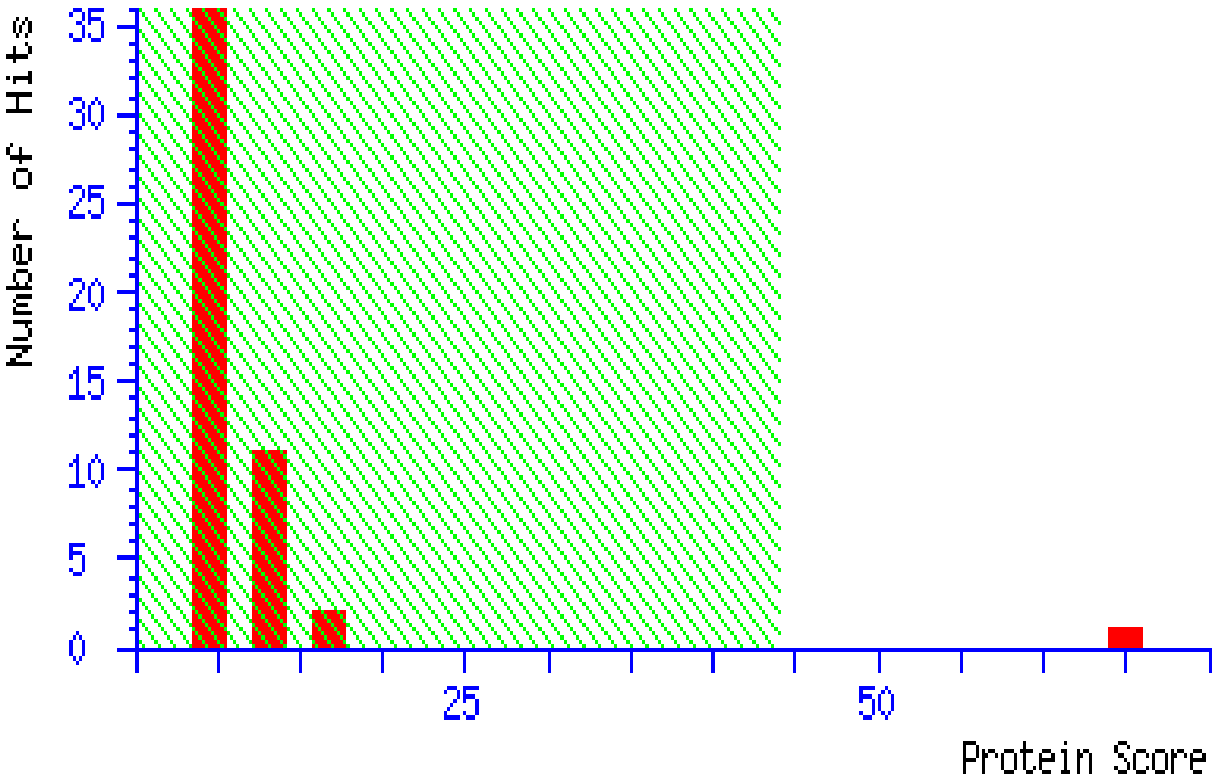


Figure 3.7: Mascot, a histogram of the probability based score results for protein identification from sample 10.

Mascot search results for protein spot (from sample 10) that was excised then run via MALDI TOF analysis for identification. The ion score is $-10^{x}\text{Log}(P)$ where P is the probability that the match observed is a random event. Scores > 44 indicates extensive homology ($p<0.05$). The protein hits identified it as K2C1, Keratin, Homosapiens; Mass: 66170, Score: 65 which indicates that contamination may have occurred.

3.4 Discussion

We sought to identify the glycoprotein that positively stained with PAS previously was AGP (Chirwa et al. 2012) using identification by MALDI-TOF-TOF. Firstly we purified the gastric scrapes and we observed typical mucin peaks after isopycnic density gradient ultracentrifugation with 4M CsCl and GuHCl (Marshall et al. 1978; Carlstedt et al. 1983b).

The purified mucins were freeze-dried then separated by molecular weight on a 1D SDS-PAGE. Only samples from patients 2, 4, 8, 9 and 10 (five out of ten patients) stained positively for PAS indicating the presence of glycoprotein at approximately 45-50kDa. It is unclear why not all mucus scrapes contain this glycoprotein but one may speculate that this may be due to various medications that differ from patient to patient during their hospital stay. AGP has the ability to bind to basic and neutral drugs such as tamoxifen, a well-known cancer drug (Schmid et al. 1973). This may result in differences in the regulation of the glycoprotein resulting in down regulation in some patients and up regulation in others. It may also be due to inter-individual variation in the expression of the same protein.

Samples 4, 9 and 10 were then separated two-dimensionally and each stained with PAS and Coomassie Brilliant Blue. PAS staining was performed to positively identify the glycoprotein (Mantle et al. 1978) . Coomassie Blue staining was used as it is highly compatible with MALDI-TOF-TOF MS analysis (Nishihara et al. 2002) which would allow identification of the unknown glycoprotein. Samples 4 and 9 has higher staining intensity with both PAS and the Coomassie Blue stain indicating high levels of the glycoprotein unlike sample 10 which had low staining and thus low expression. We observed a high amount of protein spots in sample 4 but observed less in sample 9 and even less in sample 10. This may be attributed to medication that patient 6 and 9 were administered or immune suppression corresponding to the stage of disease resulting in the decrease in overall protein expression or it may again be inter-individual variation.

Unlike previous work in our laboratory that showed four protein spots after staining with Coomassie Blue (Chirwa et al. 2012), we observed one pink PAS spot (Figure 3.3) and one Coomassie Blue spot (Figure 3.4a-c) within the pI range pH 3-4 and molecular weight range between 45-50 kDa. The reason for the difference in results is unknown but it may be due to different glycoforms of AGP due to inter-individual variation or stage of disease (Fournier et al. 2000) .

The protein spot of interest was excised from the gel, digested with trypsin and peptide fragments formed were identified by MALDI-TOF-TOF MS. The protein spot of interest in sample 4 and 9 was identified as alpha-1-acid glycoprotein (Homo sapiens). Sample 9 had a high protein MOWSE score of 71 compared to sample 4's score of 49. This may be attributed to higher expression of AGP in sample 9. Both results were found to be significant ($p<0.05$) thus we confirmed the previous findings that the pink spot that fragmented with MUC5AC is AGP (Mall et al. 1999; Chirwa et al. 2012). This also supported previous findings that the protein was N-linked (Mall et al. 1999). Sample 10 was identified as keratin indicating contamination had occurred prior to MALDI-TOF-TOF identification.

The theoretical monoisotopic mass of AGP identified in using the Mascot search engine was 23.7kDa but the actual molecular weight is 45kDa. Mass spectrometry detects peptides from a database and identifies the protein of interest by matching protein sequences. The theoretical mass therefore represents the largest peptide that was used to identify the protein (Gygi et al. 1999).

Chapter four - The development of an ELISA for AGP and mucin detection

4.1 AGP Blood Plasma Concentration

4.1.1 Healthy Patients compared to Cancer Patients

Blood samples were collected from gastric cancer patients with irresectable tumours three days after a gastrectomy had been performed. The healthy control samples were collected in the laboratory from volunteers. The plasma was separated from the blood cells and used in an ELISA kit for the detection of AGP. This was done to compare the levels of AGP in healthy controls and cancer patients and ascertain if there were any significant differences in AGP concentration between the two groups.

Statistical analysis was performed using the StatPlus package from Analystsoft. Standard deviations were calculated as for a sample population. The alpha level of significance was chosen at 0.05 and *p*-values were calculated using standard homo- and heteroscedastic t-tests dependant on differences in variance.

Figure 4.1 shows the AGP concentrations between the two groups. The average concentration of AGP in the control samples was 547µg/ml (standard deviation of 142) which was higher than the average concentration in the cancer samples which was 336µg/ml (standard deviation of 102). The *p*-value was 0.00006.

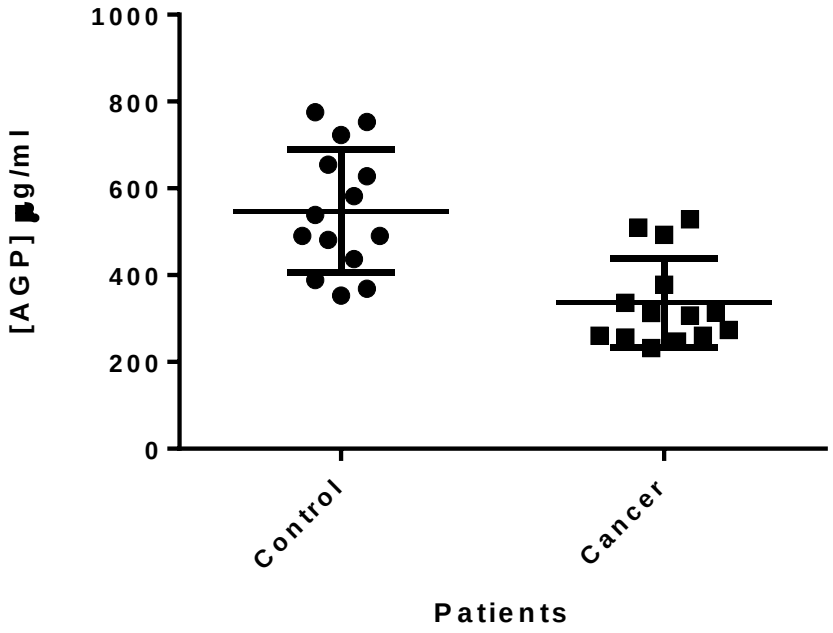


Figure 4.1: Human blood plasma concentration of AGP of healthy controls and cancer patients.

Concentrations of AGP were determined using an alpha-1-acid glycoprotein ELISA kit (Abcam). Standards provided by the kit were used to generate a standard curve and this was used to determine the AGP protein concentrations in each blood plasma sample from healthy controls and cancer patients. The average AGP concentration in the healthy controls was 547µg/ml and in the cancer patients was 336µg/ml (*p*-value of 0.00006).

4.2 Determination of Mucin Blood Plasma Levels

Blood samples were collected as described earlier and the plasma separated by centrifugation. A semi-quantitative sandwich ELISA was used to determine the levels of the four gastric mucins which are MUC1, MUC4, MUC5AC and MUC6 (measured at OD 405nm). We sought to evaluate if there were any detectable levels of each mucin in blood plasma and determine any differences between gastric cancer patients and healthy controls.

4.3 MUC1 Blood Plasma Levels

4.3.1 Healthy Controls compared to Cancer Patients

Figure 4.2 shows that MUC1 levels in the plasma followed a similar trend to AGP. The control samples were notably higher than the cancer samples. The average absorbance of MUC1 in the control samples was 0.324nm, higher than the average of the cancer samples, 0.257nm. The *p*-value for this result was 0.00033.

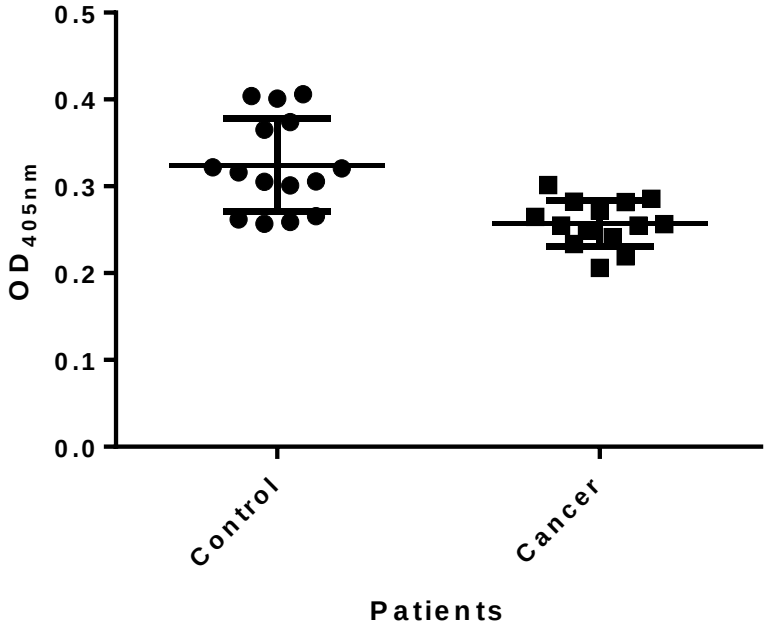


Figure 4.2: Human blood plasma levels of MUC1 of healthy controls and cancer patients.

MUC1 plasma levels were determined using a semi-quantitative sandwich ELISA. The levels of MUC1 in healthy controls were compared to cancer patients. The average MUC1 absorbance levels in healthy controls is 0.324nm and in cancer patients 0.257nm measured at an OD of 405nm (*p*-value=0.00033).

4.4 MUC4 Blood Plasma Levels

4.4.1 Healthy Controls compared to Cancer Patients

Figure 4.3 shows the blood plasma levels of MUC4 from healthy controls and cancer samples. The average absorbance for the controls was 0.265nm which was only slightly higher than the cancer samples whose average measured at 0.260nm. The *p*-value was 0.343.

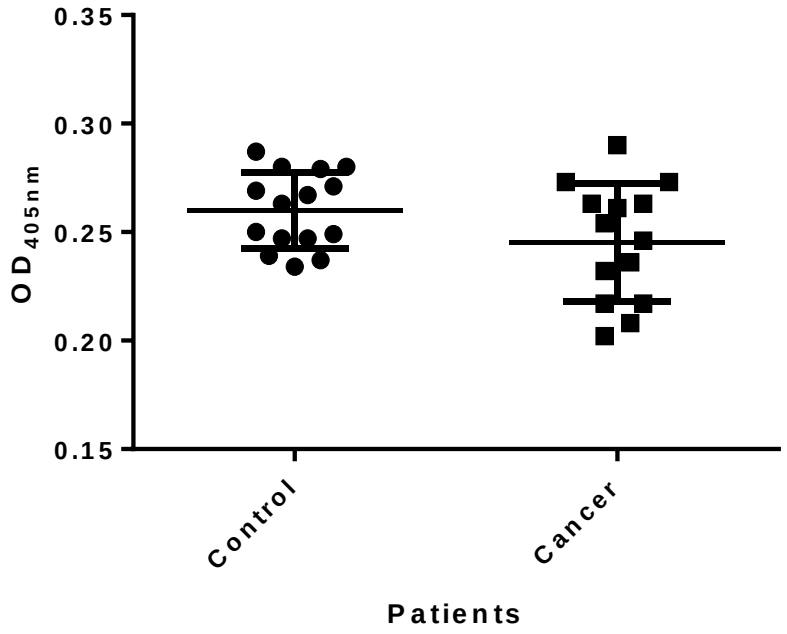


Figure 4.3: Human blood plasma levels of MUC4 of healthy controls and cancer patients.

MUC4 plasma levels were determined using a semi-quantitative sandwich ELISA. The levels of MUC4 in healthy controls were compared to cancer patients. The average MUC4 levels in the healthy controls had an OD of 0.265nm and cancer patients had an OD of 0.260nm measured at 405nm (*p*-value=0.343).

4.5 MUC5AC Blood Plasma Levels

4.5.1 Healthy Controls compared to Cancer Patients

MUC5AC absorbance levels are shown below in Figure 4.4. The average absorbance for the control samples was 0.301nm which was higher than the average of the cancer samples at 0.261nm. The *p*-value was 0.023.

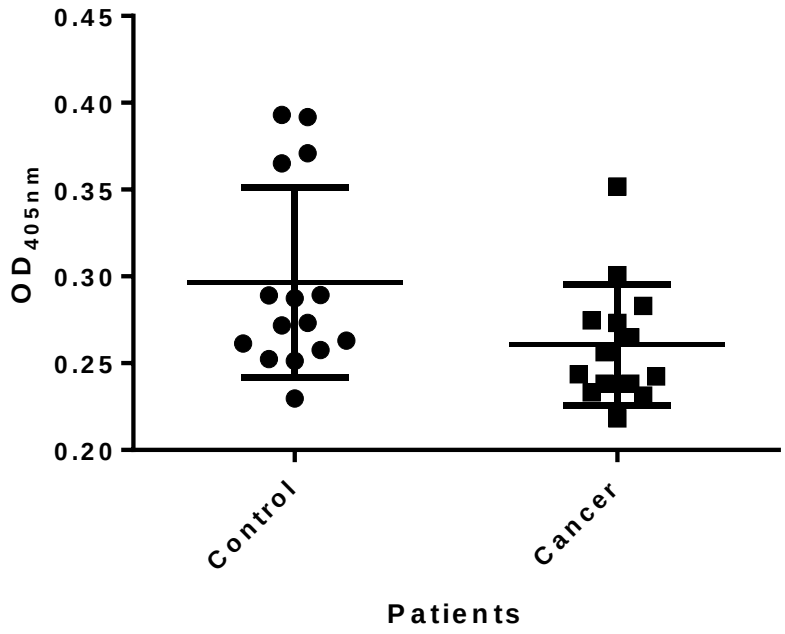


Figure 4.4: Human blood plasma levels of MUC5AC of healthy controls and cancer patients.

MUC5AC plasma levels were determined using a semi-quantitative sandwich ELISA. The levels of MUC5AC in healthy controls were compared to cancer patients. The average MUC5AC levels in the healthy controls had an OD of 0.301nm and in cancer patients the OD was 0.261nm (*p*-value=0.023).

4.6 MUC6 Blood Plasma Levels

4.6.1 Cancer Patients compared to Healthy Controls

Figure 4.5 shows the absorbance levels of MUC6 in controls and cancer samples. We observed that MUC6 levels had a similar trend to MUC4 levels. There was a minimal difference in plasma levels between the two groups. The average absorbance for the control samples was 0.298nm which was slightly higher than the average of the cancer samples at 0.295nm. The *p*-value was 0.379.

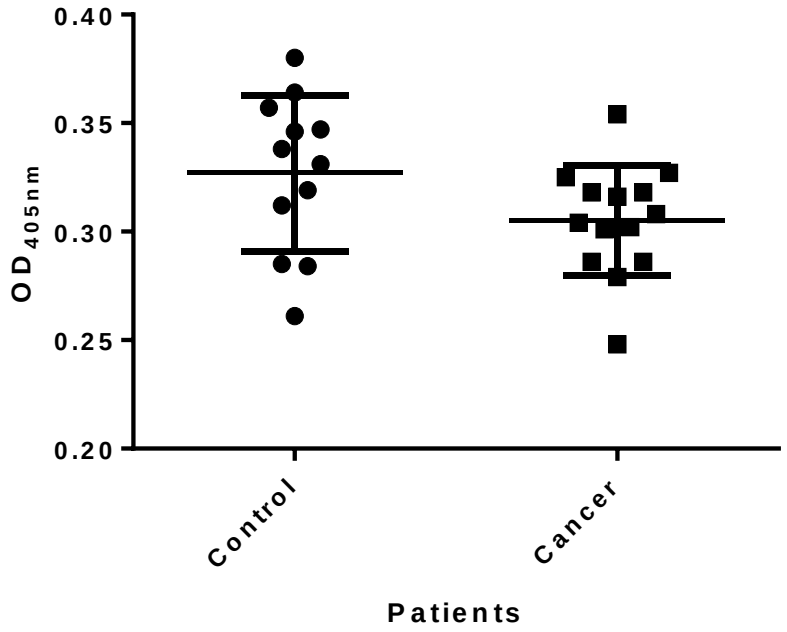


Figure 4.5: Human blood plasma levels of MUC6 of healthy controls and cancer patients.

MUC6 plasma levels were determined using a semi-quantitative sandwich ELISA. The levels of MUC6 in healthy controls were compared to cancer patients. The average MUC6 levels in the healthy controls had an OD of 0.298nm and cancer patients had an OD of 0.295nm measured at 405nm (*p*-value=0.379).

4.7 Discussion

The concentration of AGP was determined from the blood plasma of healthy controls and compared with gastric cancer patients. The average concentration of AGP found in the controls was 547µg/ml which was higher than the average of the patients which was 336µg/ml (*p*-value=0.00006). This is very surprising as AGP is an acute phase protein which increases in response to inflammation which would be present in gastric cancer patients. Work done in other laboratories has confirmed this as they observed an increase in AGP blood plasma concentration in liver (Kim et al. 2006) breast, lung (Hashimoto et al. 2004) and ovarian cancer (Abramson et al. 1982; Duché et al. 2000). Other laboratories also showed that patients with malignant disease of the gastrointestinal tract had elevated AGP plasma levels compared to healthy controls (Szilvas et al. 1998) . We expected to observe similar results but the AGP concentration was found to be higher in the control group. The reason for this contradiction in our results is unclear but we speculate that it is probably the advanced stage (non-curable resection) of the patients' disease that leads to immune suppression (Ben-Baruch 2006). Inflammation aids tumour progression (Coussens et al. 2002) and an immune deficiency may result in the down-regulation of cytokines that stimulate production of acute phase proteins such as AGP (Daveau et al. 1994) , which would normally combat and reduce inflammation (Fournier et al. 2000) .

Similar to the trend observed with AGP, the MUC1 blood plasma levels detected in the control group were higher than in the cancer group (*p*-value=0.00033). This is in line with previous work that observed a decrease in MUC1 in cancer patients. This is due to aberrant glycosylation of MUC1 which results in shortened glycoforms that aid in neoplastic transformation (Baldus et al. 2004; Bafna et al. 2010). Previous work in our laboratory observed an over-expression of MUC1 in gastric cancer tissue (Chirwa et al. 2012).

The blood plasma levels of MUC4 in both groups were very similar and therefore no difference was observed between the two groups (*p*-value=0.343). MUC4 expression has been shown to be up-regulated in gastric tissue of adenomacarcinoma patients (López-Ferrer et al. 2000; Senapati et al. 2008) but we observed no significance difference in the blood plasma results. This may be the result of low levels of MUC4 in the plasma as it is a transmembrane mucin and thus no difference could be detected.

The levels of MUC5AC in the blood plasma were measured. We found a statistically significant difference between the control group which measured higher levels of MUC5AC than the gastric cancer group (*p*-value=0.023). This result is surprising as MUC5AC is known to decrease in gastric cancer (Corfield et al. 2000).

Similar to MUC4, we observed no significant difference in MUC6 plasma levels between the control and cancer group (*p*-value=0.379). This is also surprising as MUC6 normally decreases in gastric cancer and relates closely to the tumour progression (Corfield et al. 2000).

The results we observed could be confirmed by an increased sample size of both the control and cancer group. This would help determine if the differences observed or lack thereof is due to inter-individual differences or changes in protein levels in the blood plasma. This would also help ascertain if a mathematical model could be developed and aid in the development of a gastric disease biomarker.

Chapter five – Determination of protein expression in gastric tissue

5.1 Determination of Immunogenic score in Gastric Tissue using Immunohistochemistry

Formalin fixed paraffin wax embedded tissue blocks were obtained from the archives of the Division of Anatomical Pathology, National Health Laboratory Service, Groote Schuur Hospital (n=4) from patients who had undergone partial or total gastric resection. Histochemistry techniques were used to confirm the presence of mucins in the tissue. Immunohistochemistry techniques were employed using antibodies against AGP, MUC1, MUC1core (MUC1c), MUC2, MUC4, MUC5AC and MUC6 to determine protein expression within the gastric tissue. MUC1c and MUC2 were included as changes in expression of MUC1c have been observed in gastric cancer tissue. MUC2 was assessed in the tissue as *de novo* expression is observed in gastric cancer tissue. Table 5.1 and Figure 5.1 show a summary of the immunohistochemical score given to each gastric tissue section for each target antigen.

Antigen	**Study patient number**			
	3	**4**	**10**	**14**
AGP	0	1+	0	3+
MUC1	0	3+	2+	1+
MUC1c	0	2+	3+	4+
MUC2	3+	0	1+	0
MUC4	0	1+	3+	0
MUC5AC	2+	0	0	3+
MUC6	2+	0	0	0

Table 5.1: Immunohistochemical score for gastric tissue staining from Patients 3, 4, 10 and 14.

A semi-quantitative method was used to determine the immunohistochemical score whereby sections were examined at (x40 objective) and scored: <5% non stained cells = 0; 5-25% stained cells = 1+; 26-50% stained cells = 2+; 51-75% stained cells = 3+; >75% stained cells = 4+.

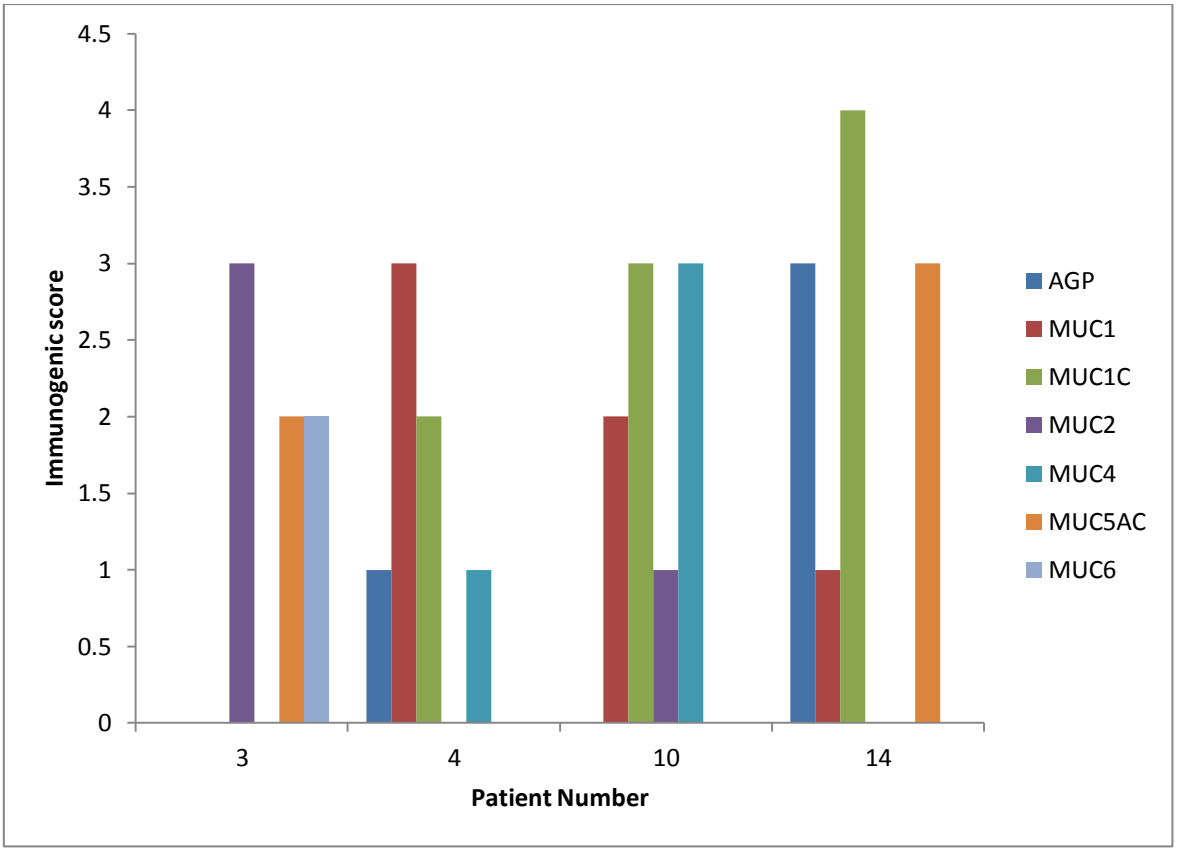


Figure 5.1: Immunohistochemical score showing differences in AGP and mucin expression in gastric tissue.

Gastric tissue from patients 3, 4, 10 and 14 were each stained separately with AGP, MUC1, MUC1c, MUC2, MUC4, MUC5AC and MUC6 using standard immunohistochemistry techniques.

5.2 Protein Expression in Gastric Tissue

The expression of AGP, MUC1, MUC1c, MUC2, MUC4, MUC5AC and MUC6 was determined using immunohistochemistry techniques. Three types of gastric tissue were stained for each antigen, namely: normal gastric, intestinal metaplasia (IM) and adenocarcinoma (AD) tissue.

5.2.1 AGP expression in gastric tissue

Figure 5.2 shows images of the AGP staining in gastric tissue. Figure 5.2A shows extensive staining in normal gastric tissue within the parietal cells. Figure 5.2B shows strong staining (score 3+) in the columnar cells surrounding the goblet cells (shown by the arrow) in the IM tissue. We only observed staining in one of the three AD patients. Unlike the other two areas of gastric tissue, the AD (shown in Figure 5.2C) tissue showed very low staining of AGP, immunogenic score 1+, indicating low level of expression in the tumour cells.

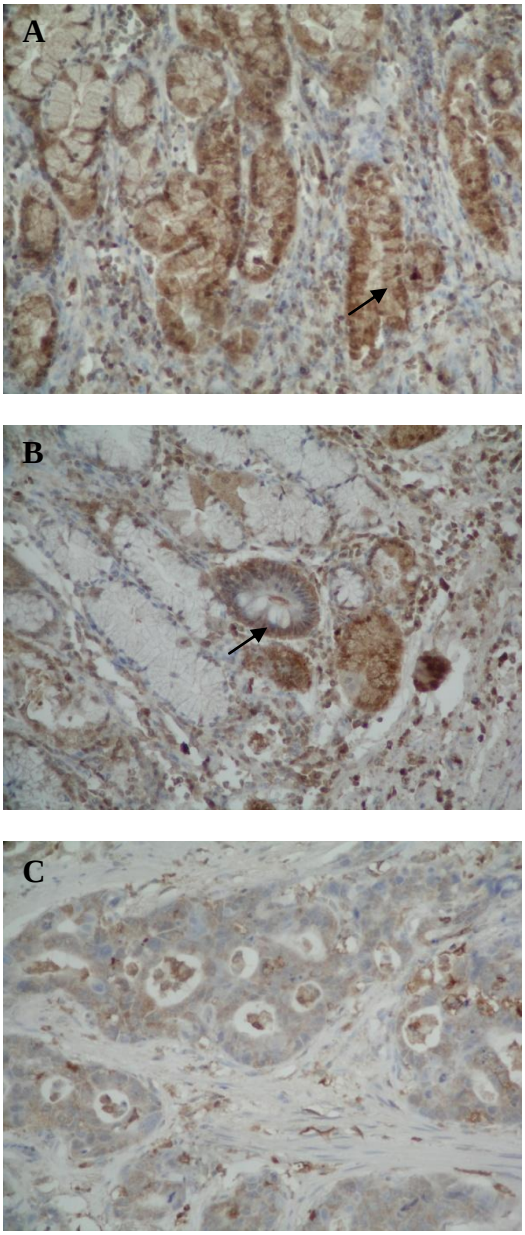


Figure 5.2: Immunohistochemical AGP staining of three different sections of gastric tissue.

A: Normal gastric tissue showing strong expression in the parietal cells (shown by the arrow). **B:** Intestinal metaplasia tissue showing high expression in the columnar cells (shown by the arrow). **C:** In the adenocarcinoma tissue there was minimal staining in the tumour cells (x80).

5.2.2 MUC1 expression in gastric tissue

In Figure 5.3A, there are isolated cells showing MUC1 staining (less than 5%) within the normal gastric tissue which is regarded as a negative result. Figure 5.3B shows no staining (score of 0) in IM tissue indicating no MUC1 expression. In Figure 5.3C and 5.3D staining is seen in two of three AD patients within the cells and glands of AD tissue. Figure 5.3C shows high levels of expression in the membranes of the malignant cells resulting in a score of 3+. Glandular lumen staining was observed in the AD tissue shown in Figure 5.3D and it was given a score of 2+.

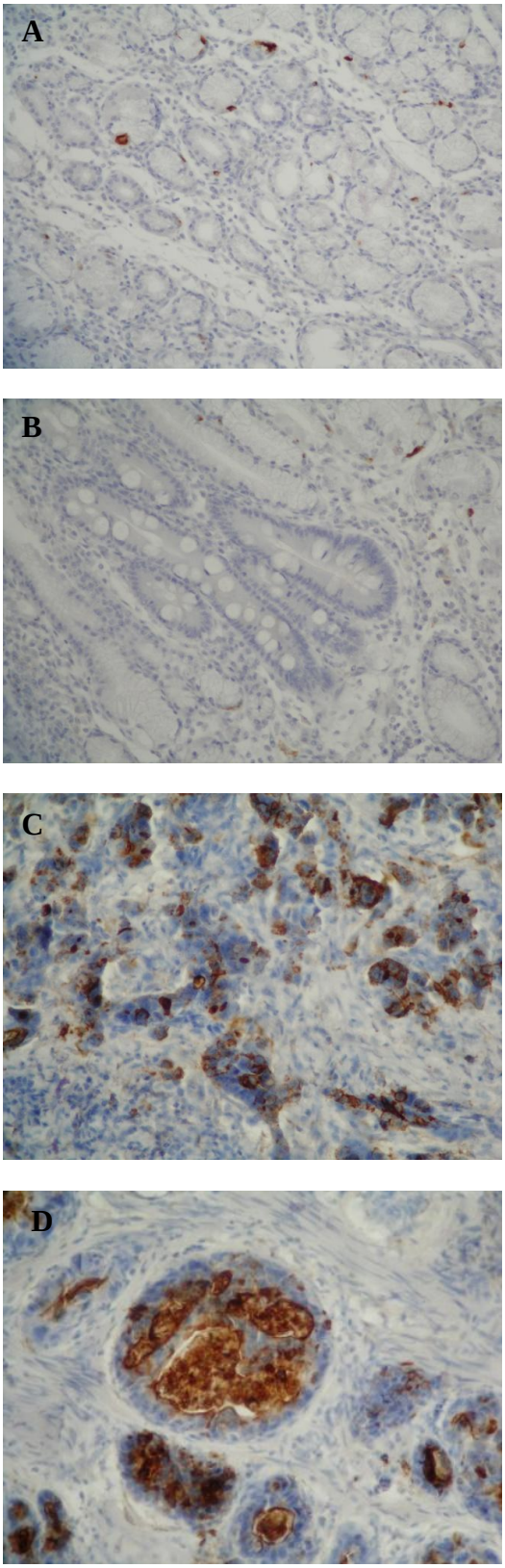


Figure 5.3: Immunohistochemical MUC1 staining of three different sections of gastric tissue.

A: Isolated cells expressed MUC1 in the normal gastric tissue. **B:** Intestinal metaplasia tissue showed no staining. **C:** High levels of cellular membrane expression in the tumour cells of the adenocarcinoma tissue. **D:** High levels of expression in the lumen of the malignant glands (x80).

5.2.3 MUC1C expression in gastric tissue

Our results shown in Figure 5.4 indicate that MUC1c expression is similar to the MUC1 expression observed in Figure 5.3. There was less than 5% staining observed in the normal gastric tissue in Figure 5.4A indicating a negative result and a score of 0. Figure 5.4B shows the IM tissue where no MUC1c staining was observed. We observed MUC1c staining in two of the three AD patients. The AD tissue shown in Figure 5.4C was scored of 2+. High levels of staining were also observed within the lumens of glands of the AD tissue shown in Figure 5.4D (score +3).

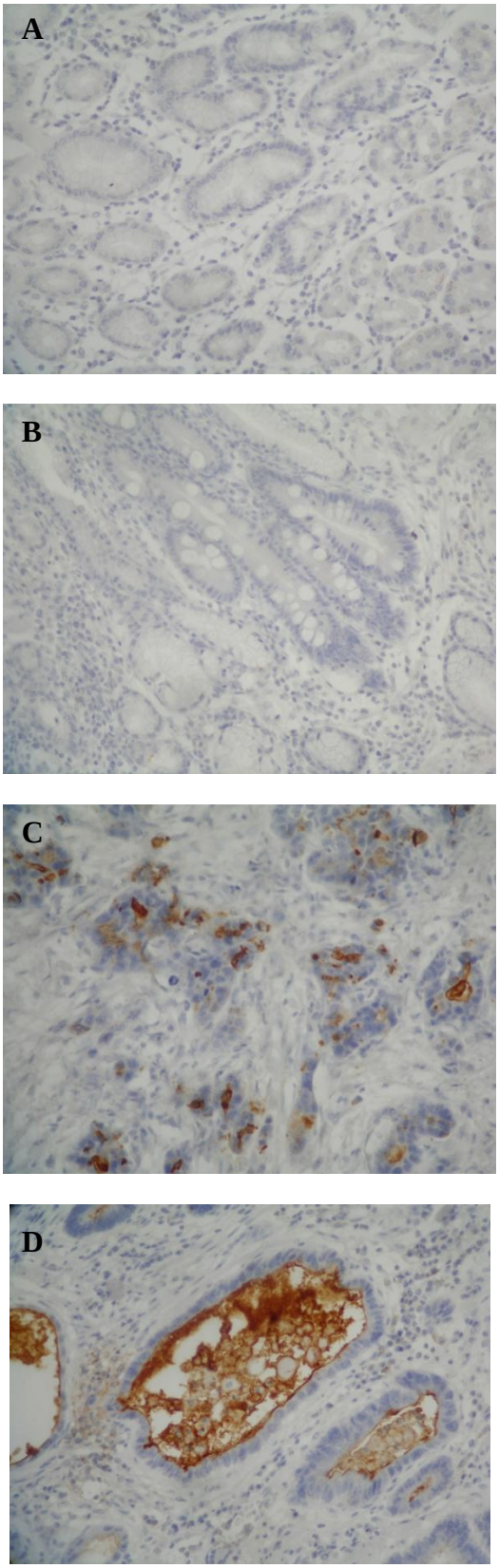


Figure 5.4: Immunohistochemical MUC1C staining of three different sections of gastric tissue.

A: Minimal staining was observed in the normal gastric tissue **B:** Intestinal metaplasia tissue showed no staining of MUC1c. **C:** Cellular staining was observed in the adenocarcinoma tissue. **D:** Adenocarcinoma tissue also showed luminal gland staining (x80)

5.2.4 MUC2 expression in gastric tissue

Figure 5.5 represents MUC2 expression. In the normal gastric tissue, there was no MUC2 staining (Figure 5.5A). Figure 5.5B indicates low levels of MUC2 expression on the membranes of the goblet cells in the IM tissue (shown by an arrow). Two of the three AD patients had MUC2 expression. We observed the highest level of cellular expression in Figure 5.5C and this AD tissue section was given a score of 3+.

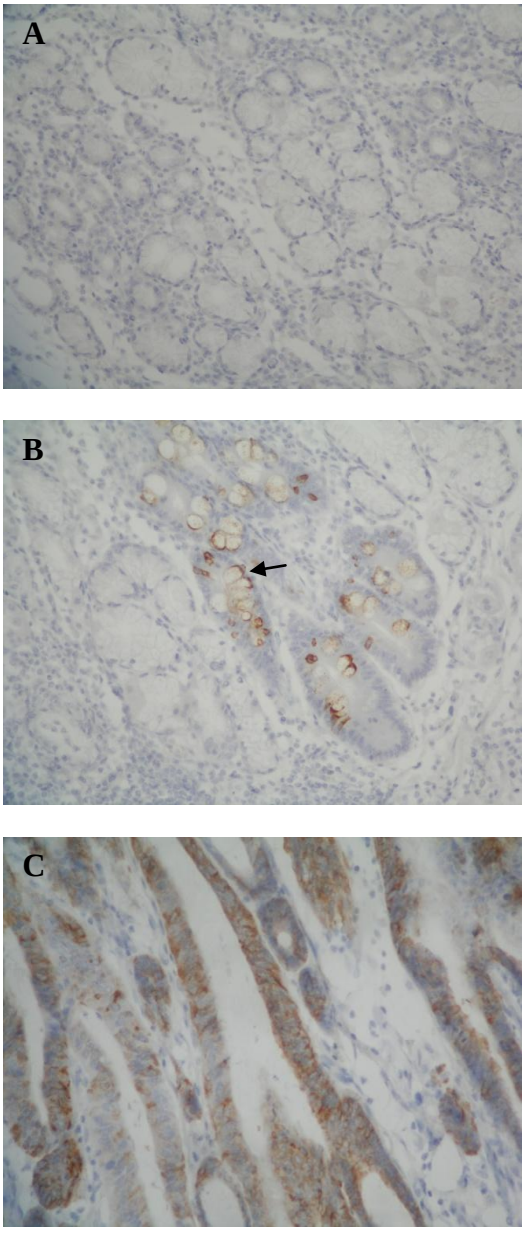


Figure 5.5: Immunohistochemical MUC2 staining of three different sections of gastric tissue.

A: No staining was observed in the normal gastric tissue **B:** Intestinal metaplasia tissue showed focal staining of the membrane of the goblet cells. **C:** Adenocarcinoma tissue showed high levels of cellular staining (x80).

5.2.5 MUC4 expression in gastric tissue

In normal gastric tissue, shown in Figure 5.6A we observed staining of the cytoplasm of cells indicating the presence of MUC4. Focal staining was observed in the areas with IM tissue (shown in Figure 5.6B) and this section was given a score of 0. The AD tissue showed very high levels of MUC4 cellular staining (one out of three patients) and was given an immunogenic score of 4+.

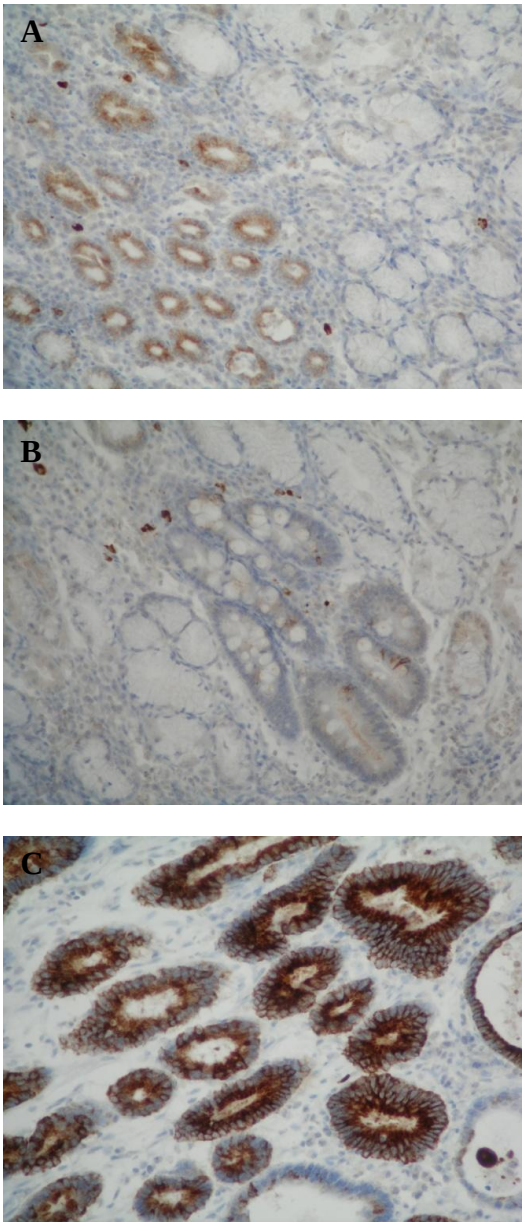


Figure 5.6: Immunohistochemical MUC4 staining of three different sections of gastric tissue.

A: Staining was observed in the cytoplasm of cells within the normal gastric tissue **B:** Intestinal metaplasia tissue showed very low levels of expression. **C:** Adenocarcinoma tissue showed high levels of cellular staining (x80).

5.2.6 MUC5AC expression in gastric tissue

We observed high levels of MUC5AC staining in the glandular cells of normal gastric tissue shown in Figure 5.7A. Figure 5.7B shows no MUC5AC staining in the IM tissue although surrounding normal gastric tissue was stained. Staining was observed in two of the three cases of adenocarcinoma. Figure 5.7C shows positive staining from one of the cases. High levels of MUC5AC membrane staining of the malignant epithelial cells of the glands (score 2+) was observed in the AD tissue, shown in Figure 5.7C.

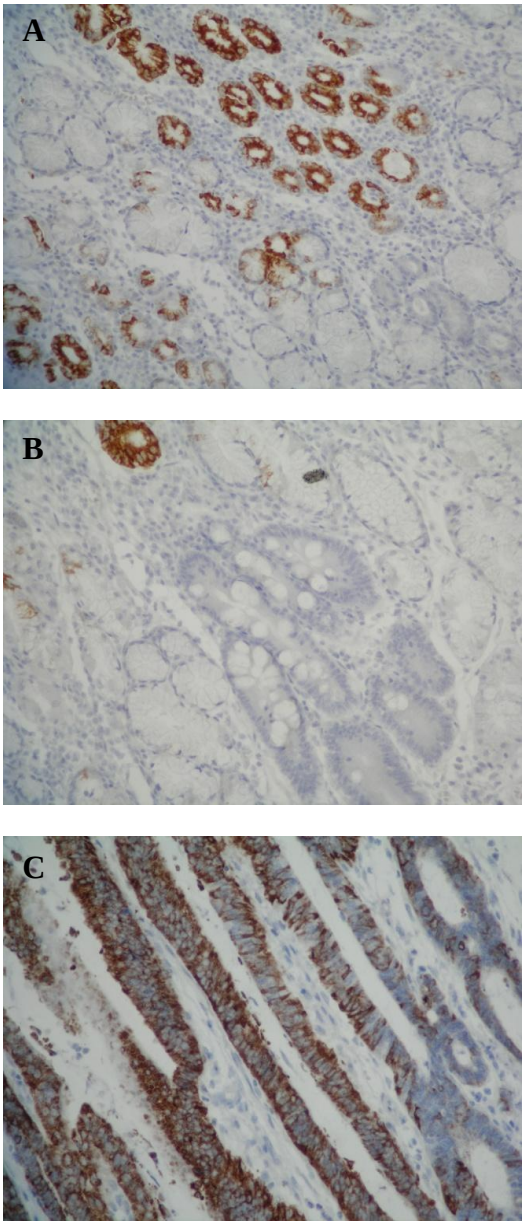


Figure 5.7: Immunohistochemical MUC5AC staining of three different sections of gastric tissue.

A: A high level of glandular cell staining was observed in the normal gastric tissue **B:** Intestinal metaplasia tissue showed no staining. **C:** Adenocarcinoma tissue showed high levels of expression in the malignant epithelial cells of the glands (x80).

5.2.7 MUC 6 expression in gastric tissue

Similar to Figure 5.7, high levels of MUC6 staining was observed on the membrane of glandular cells in normal gastric tissue, shown in Figure 5.8A. No staining was observed in Figure 5.8B in the areas with IM tissue but staining was observed in the surrounding normal tissue. High levels of staining were observed in the membranes of glandular AD tissue in Figure 5.8C (score 2+) but only one of the three adenocarcinoma cases showed MUC6 expression.

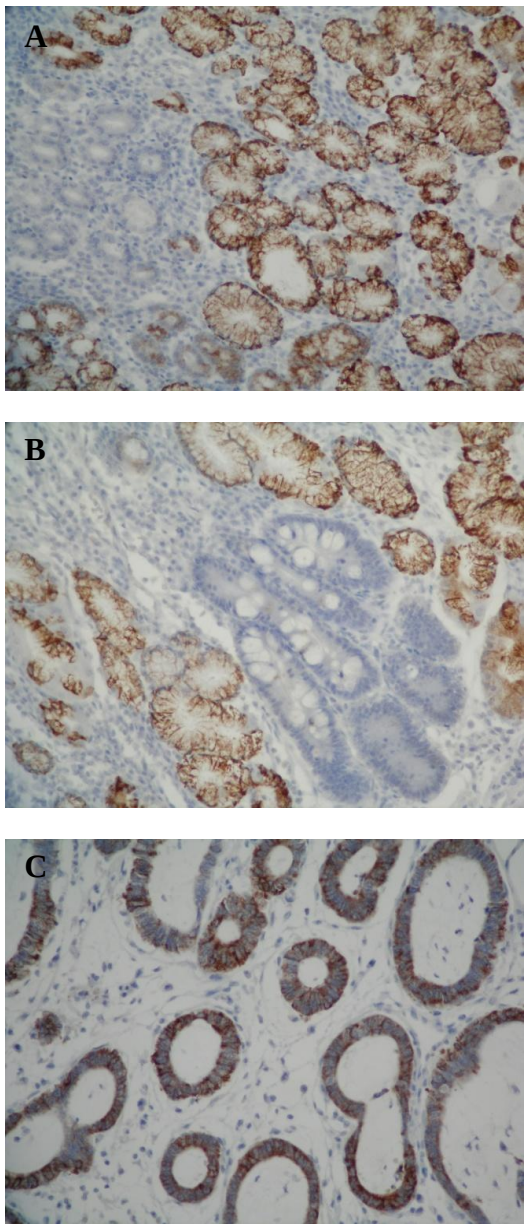


Figure 5.8: Immunohistochemical MUC6 staining of three different sections of gastric tissue.

A: A high level of glandular staining was observed in the normal gastric tissue **B:** Intestinal metaplasia tissue showed no staining. **C:** Adenocarcinoma tissue showed high levels of expression in the membranes of glandular cells (x80).

5.3 Discussion

The expression of AGP and the mucins in gastric tissue was similar to previous findings in our laboratory (Chirwa et al. 2012). AGP expression was largely in the parietal cells of the normal gastric tissue. It was highly expressed in the columnar cells surrounding the goblet cells of the intestinal metaplasia (IM) tissue. Surprisingly, the adenocarcinoma (AD) tissue lacked AGP expression with only one of the three patients staining positively for AGP. This may be attributed to high levels of neoplastic transformation in the cells resulting in the reduction of the anti-inflammatory proteins such as AGP (Daveau et al. 1994) . Extensive changes of normal gastric tissue into IM tissue may also result in a decrease of AGP expression and more tests would have to be done to confirm this. The lack of AGP expression observed in the AD tissue correlates with our ELISA results. This leads us to believe that AGP is actually reduced in gastric cancer patients compared to healthy controls and is not overexpressed as previously thought.

No MUC1 and MUC1c expression was observed in the normal gastric tissue which is surprising as it is located in the surface epithelium of neck cells. Likewise, no expression was observed in the IM tissue. It is unclear why no expression was observed in the normal tissue but the use of a different antibody targeting a different epitope may produce positive results. Likewise, this section of normal tissue may have very low undetectable levels of MUC1 and MUC1c.

High levels of expression were observed in the AD tissue for both MUC1 and MUC1c (two of three patients). Expression may not have been observed in one patient as neoplastic transformation of MUC1 may have altered the epitopes which would normally be bound by the MUC1 antibody. Our ELISA results indicated that MUC1 plasma expression was higher in the healthy controls than the cancer samples but in the tissue we observed the opposite result. As MUC1 is a transmembrane mucin, it may be easier to detect in the blood than in the deep gland cells where it is normally found in the stomach.

In line with previous work (Utsunomiya et al. 1998; Gürbüz et al. 2002) , MUC2 expression was not observed in the normal gastric tissue but *de novo* expression was seen in both the IM tissue and the AD tissue. Higher levels of MUC2 were observed in the AD tissue and this may be a result of malignant transformation compared to the IM tissue.

MUC4 expression in normal and diseased gastric tissue was identified in our laboratory using immunohistochemistry techniques (Taylor et al. 1998). Similarly, we observed very high expression of MUC4 in one patient with AD compared to normal gastric tissue or IM tissue. This result does not correlate with the findings from our ELISA. As previously explained MUC4 is a transmembrane mucin like MUC1 and may be easier to detect in the blood. In gastric cancer, MUC1 and MUC4 often have alterations in their glycosylation pattern resulting in shortened glycans which may not be recognised by the antibodies we have used in this study (López-Ferrer et al. 2000; Senapati et al. 2008). Regarding the other two AD patients, we observed low MUC4 expression in one and no expression in the other which was similar to our findings in the normal tissue. This is similar to our ELISA results where there was no significant difference between the control and cancer groups.

High MUC5AC expression was observed in the glandular cells of the normal tissue. Minimal expression was observed in the IM tissue which is in line with previous work but we observed high levels of expression in one of the three AD patients (Corfield et al. 2001; Mall 2008). This correlates with our ELISA results in which we observed similar levels of MUC5AC in both the control and cancer group. The other two AD patients had no MUC5AC staining which may be due to the progress of the disease which causes down-regulation of MUC5AC (Corfield et al. 2000) .

The expression levels of MUC6 between the normal tissue and one of the AD patients directly correlates with the levels found in blood plasma. In our ELISA results, we observed very similar levels of MUC6 between the control and cancer group. As expected no MUC6 expression was observed within the area of IM. Interestingly, two AD patients (patient 4 and 10) had no MUC5AC or MUC6 tissue staining, which may be a result of disease progression (Corfield et al. 2000) .

Discussion, conclusion and future work

According to the American Cancer Society, it is estimated than in the year 2013, approximately 1,660,290 new cancer cases were expected in the United States (www.cancer.org). Gastric cancer represents a large number of these cases as it is one of the highest leading causes of cancer-related deaths worldwide (Ferlay et al. 2010; Jemal et al. 2011). Gastric cancer has a high prevalence in the Western Cape region of South Africa and patients usually present at hospitals when the disease is already at a late stage. At this point, there are only a few treatment options available which are not always successful. The biomarkers that are currently available, for example carcino-embryonic antigen, are only detectable at the late stages of the disease (Fuchs et al. 1995). There is a need for the development of a clinical biomarker for gastric cancer which can aid in early detection. This could result in the development of a clinical detection biomarker for country wide screening for the early detection of gastric cancer. Screening programmes have proved successful in countries like Japan where research showed screening with barium X-ray examinations at five year intervals could reduce gastric cancer mortality by sixty percent (Fukao et al. 1995). Screening using photofluorography (Lee et al. 2006b) and the serum pepsinogen test (Miki 2006) in Japan has also been associated with a reduction in the gastric cancer mortality rate.

Our laboratory showed that a 55-65kDa glycoprotein reproducibly fractionated with gastric mucins from crude mucus scrapings and gastric juices from patients with ulceration, carcinoma (Mall et al. 1999) and Menetrier's disease (Mall et al. 2003). As previously described, this glycoprotein was identified as AGP (Chirwa et al. 2012) . This project sought to confirm these results using crude mucus scrapings from gastric cancer patients as well as identifying the PAS positive spot. Three samples were chosen to use for the identification of AGP. Two of these samples were positively identified as AGP and unfortunately the third sample was contaminated with keratin.

AGP is an acute phase protein which regulates phagocytic functions and T-cell action (Williams et al. 1997). It also primarily acts as an anti-inflammatory and immunomodulatory agent (Daemen et al. 2000; Hochepied et al. 2003). AGP has long been considered a possible candidate for a clinical biomarker for a range of cancers as it is usually significantly elevated in cancers (Fournier et al. 2000). Previous work has shown the successful development of a

rapid immunochromatographic strip to test asialo AGP blood plasma levels to diagnose hepatocellular carcinoma (HCC) (Lee et al. 2006a). Up to date, there has been no successful development of an AGP detection test for gastric disease. AGP has been tested with alpha-fetoprotein against HCC and the result indicated that the combination of these proteins improved sensitivity and increased accuracy in diagnosing HCC (Bachtiar et al. 2009). Kundin et. al found that AGP and pre-albumin could be used together for prostate cancer screening (Kundin et al. 1981). Consequently work has been done to assess if AGP can be used in conjunction with another protein to produce a biomarker. Mucins such as MUC1 and MUC4 have shown promise as diagnostic markers in disease (Goydos et al. 1996; Karanikas et al. 1997). The aim is to determine an ideal range of concentration of AGP in combination with a protein, such as a mucin, and establish a threshold value that would be used to detect pre-malignancy.

We assessed the possibility of AGP as a biomarker for gastric disease by determining its concentration in blood plasma as well as in gastric tissue of cancer patients. We observed that AGP was decreased (*p*-value=0.00006) in the plasma of cancer patients compared to that of healthy controls. This is surprising as AGP has been found to be elevated in numerous types of cancer including gastrointestinal tract malignancies (Szilvas et al. 1998). The majority of our sample group had irresectable (late stage) tumours thus we speculate that the reduction in AGP was a result of immune suppression (Daveau et al. 1994; Ben-Baruch 2006).

AGP binds to vitamin B_{12} (Weinstein et al. 1959) and is extensively involved in drug binding thus changes in vitamin B_{12} levels or the concentrations of compatible drugs could alter the detectable concentration of AGP in blood plasma (Fournier et al. 2000). Future work with AGP should involve measuring the levels of vitamin B_{12} and the concentration of drugs in each patient. Previous work has found that AGP levels return to baseline two to four weeks after surgery (Holley et al. 1984). We measured the concentration of AGP three days after surgery which may not represent accurate levels in gastric cancer patients.

The expression of AGP in adenocarcinoma (AD) and intestinal metaplasia gastric tissue was elevated compared to controls. Previous work in our laboratory found high levels of AGP expression in cancerous gastric tissue (Chirwa et al. 2012) thus it was surprising that only one of the three AD patients stained positively for AGP. This may be attributed to neoplastic changes within the tissue resulting in a decrease of AGP expression to undetectable levels (Daveau et al. 1994). The differences observed could also be a result of different antibodies

used in each study (which may recognise different epitopes). These contradictory results make it difficult to come to a conclusion about the correlation of AGP tissue expression and its levels in blood plasma in cancer patients.

In our study, only the clinical information for patient 4 was available for AGP identification, determination of AGP expression in blood plasma and gastric tissue. This patient underwent a full gastrectomy with a tumour that had metastasised. This may have resulted in immune suppression (Daveau et al. 1994) which we mentioned earlier, resulting in the decrease of AGP in the blood plasma.

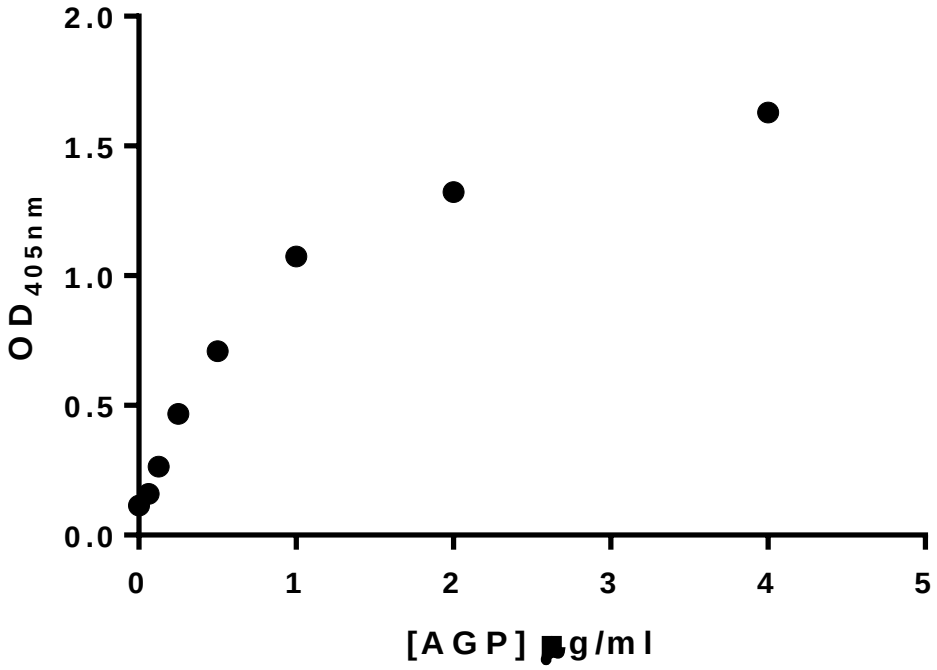
As mentioned earlier, mucins are also candidates for cancer biomarkers thus we measured the plasma levels of MUC1, MUC4, MUC5AC and MUC6 using an ELISA. MUC1 plasma levels were lower in the control group than the cancer group (p-value=0.00033) showing a similar trend to AGP. AD tissue, where two out of three patients had MUC1 expression higher than that of the normal control gastric tissue, showed a similar trend to the MUC1 blood plasma result. The significant differences observed with AGP and MUC1 make them possible candidates to be used together as a clinical biomarker. In our study, we only determined the difference of MUC1 levels between healthy controls and that of cancer patients, and not the exact concentration. Further work needs to be done to determine the concentration of MUC1 in blood plasma to accurately compare it to AGP.

De novo MUC2 expression was observed within the neoplastic tissue agreeing with Utsunomiya's results (Utsunomiya et al. 1998). It would be interesting to determine if this mucin can be detected in the blood plasma of cancer patients and this could be a study for future works. MUC4 tissue expression was similar to our ELISA blood plasma results where no difference was observed between the control and the cancer group. Interestingly, MUC6 expression in the gastric tissue of one of the AD patients correlated with our ELISA blood plasma results. The other two AD patients showed no MUC5AC and MUC6 staining we speculate this may be due to disease progression (Corfield et al. 2000). This result correlated with our ELISA for MUC5AC as the levels in the control group were higher than the cancer group (p=0.023).

Further work has to be done to determine the accuracy and sensitivity of AGP as a clinical biomarker. The combination of MUC1 and AGP may result in a candidate for a clinical biomarker. This study was subject to the number of patients who underwent surgery, but this number was low as most of the patients presented with irresectable tumours at the time of the

study and only blood samples could be collected from this group. Further work needs to be done with a larger sample size of controls and cancer patients with full clinical information to allow an accurate determination between AGP in the blood plasma and gastric tissue. AGP blood plasma levels need to be measured at regular intervals over a two-four week period to determine an accurate range in concentration in cancer patients post-surgery. The levels of AGP must also be determined in patients with other gastric diseases during the course of infection. Previous work has shown expression of genetic variants of AGP can change depending on the type of cancer (Duché et al. 2000). Further work has to be done to determine if certain glycoforms of AGP are more suitable biomarkers than others.

Appendix



Standard curve generated from the reagents provided from the AGP ELISA kit (abcam). This was used to calculate the AGP concentration in blood plasma in cancer patients and healthy controls.

REQUEST FOR BLOOD AND GASTRIC SCRAPINGS FOR BIOCHEMISTRY AND GASTRIC TISSUE TO STUDY GASTRIC MUCUS IN DISEASE

Research Laboratory
Division of General Surgery
OMB Groote Schuur Hospital
UCT Medical School, Observatory 7925

Tel: (021) 406 6168/6227 Fax: (021) 448 6461

Please fill in all the information requested:

Folder No. _____

Sex: M ☐ F ☐

Date of Birth Year: _____ Month: _____ Day: _____ Ethnic Origin: _____

Contact Address: Hospital/Clinic where samples are taken

Town: _____ Fax: _____

Tel: _____

For Laboratory use only:

Date Received: YY _____ DD _____ Computer Index No.: _____

CONSENT FOR SALIVA, BLOOD AND GASTRIC JUICE AND BUCCAL SWAB

1. I, _____ give permission for my gastric/colonic scrapings to be taken for research purposes in the investigation of disease of the stomach.
2. I, _____ give permission for my blood and a tiny piece of tumour tissue to be taken for research purposes in the investigation of stomach disease.

3. I give permission that a portion of the samples be stored indefinitely for:
    a) possible re-analysis;
    b) research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain confidential.

1. I authorize / do not authorize my doctor(s) (DELETE WHERE NOT APPLICABLE) to provide relevant clinical details to the researchers.

ALL OF THE ABOVE HAS BEEN FULLY EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED

Signature……..

Please note that your HIV status is **not** recorded on this form.

- The Human Research Ethics Committee is contactable at 021-4066338
- Please note that you are **entitled to withdraw** from the study at any time that you so wish to. This will in no way jeopardize your care as a patient
- You may not wish your samples to be re-used for analysis. If so, please sign in the space provided below

I hereby declare that I do not wish my samples to be stored for re-use in the future for analysis

Signature……..

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